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THE EFFECT OF DIABETES, PHYSICAL TRAINING, AND MAGNESIUM REGULATION ON ATPASE ACTIVITY IN THE HEART

by

© MARIE ROSSITER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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submitted by Marie Rossiter

in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Diabetes is characterized by depressed cardiac functional properties attributed to altered Mg^{+2} control resulting in a lowered $Ca^{+2}Mg^{+2}$ myofibril ATPase activity of cardiac tissue. Thus, the purpose of this study was to observe if the cellular changes associated with diabetes can be ameliorated with training. Diabetes was induced with a single I.V. injection of streptozotocin (60 mg/kg). Blood and urine glucose concentrations were 802 $\pm$ 44 and 6,965 $\pm$ 617 mg/dl respectively. The training control (TC) and training diabetic (TD) animals were made to swim ($\pm$3% body weight) 4 days/week for 8 weeks. Cardiac myofibril ATPase at 10 uM free Ca^{+2} and increasing Mg^{+2}, concentrations were reduced by 45, 45, and 50% in the sedentary diabetics (SD) compared to sedentary control animals (SC) ($p<0.05$). Swim training enhanced the ATPase activities at all Mg^{+2} levels for TC while the myofibril ATPase activity at all Mg^{+2} concentrations was not altered in the TD group. The Mg^{+2} stimulated myofibril ATPase activity was similarly reduced in diabetics at all Mg^{+2} concentrations ($p<0.05$). Increasing Mg^{+2} concentrations (0.04-10.0 mM) resulted in an elevated ATPase activity for SC and depressed ATPase activity in TD animals and had no effect on the cardiac myofibril ATPase activity for TD or SD. The results suggest that the depressed myofibril ATPase activity of diabetic hearts is a function of Mg^{+2} regulation, and that swimming does not alleviate this depression.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
METHODOLOGY	5
Animal Care	5
Experimental Design	5
Tissue Sampling	6
Myofibril Isolation	6
Myofibril ATPase Determination	7
Calcium Binding	7
Limitations of the Study	10
Definitions	10
RESULTS	12
DISCUSSION	19
BIBLIOGRAPHY	27
APPENDICES	59
A. Review of Literature	59
Structure	60
Function	94
Regulation	130
Cardiovascular Adaptations With Training	151
Diabetes	158
B. Biochemical Procedures and Chemicals	171
C. Raw Data	180
D. Statistical Procedures	187

LIST OF TABLES

Table	Description	Page
I	Training Schedule of Endurance Trained Rats	9
II	General Features of the Experimental Group	15
III	Calcium Binding Data	15

LIST OF FIGURES

Figure		Page
1.	Ca^{+2}-Mg^{+2} Myofibril ATPase with Diabetes	16
2.	Mg^{+2} ATPase of Control and Diabetic Cardiac Muscle	17
3.	Ca^{+2}-Mg^{+2} Stimulated ATPase Activity in Control, Diabetic and Trained Hearts.	18
4.	Refractory State Model	112

INTRODUCTION

The greater incidence of myocardial infarction in controlled diabetes mellitus has traditionally been considered to be due to an enhanced degree of atherosclerotic plaque formation in the coronary arteries (Kessler, 1971; Braunwald, 1980). However the results of recent investigations suggest that a primary myocardial defect may be involved particularly in light of the observation that cardiac dysfunction and failure may occur in the absence of significant atherosclerosis (Vihert et al, 1969; Ledt, 1968; Ledt, 1976; Regan et al, 1974; Regan et al, 1977). Epidimological (Kannel, 1978), clinical (Regan et al, 1977; Ahmed et al, 1975; Rubler, 1976) and pathological (Hamby et al, 1974) studies suggest the existence of a diabetic cardiomyopathy independent of atherosclerosis, coronary artery disease, hypertension, or valvular disease.

The cardiomyopathy of diabetic patients without occlusive lesions results in a reduced stroke volume and increased endiastolic pressure, concomitant with an increased stiffness of the ventricular walls (Regan et al, 1974; Regan et al, 1977). With streptozotocin induced diabetes a prolongation of isometric relaxation time, a decrease in the negative rate of ventricular pressure change (dp/dt), and a delay in reaching peak isometric and isotonic relaxation rates have also been observed (Strobek et al, 1979; Fein et al, 1980; Penparkgul et al, 1980b; Ingelbretson et al, 1980).

Since there is a positive relationship between myosin ATPase activity and the velocity of ventricular muscle contraction, these physiological alterations may be related to biochemical indices of contraction (Hamrell and Low, 1978). Biochemical evidence indicates that cardiac tissue from

diabetic animals show depressed myosin, actomyosin, and myofibril ATPase activity (Malhotra et al, 1981; Dillman, 1980; Pang and Weglicki, 1980; Pierce and Dhalla, 1981), but whether this depressed ATPase activity is due to an alteration in the structural and or regulatory factors associated with the ATPase activity is speculative.

Since the dependency of cardiac myofibril ATPase activity on free Ca^{+2} levels is not altered with diabetes (Pierce and Dhalla, 1981) a possible explanation for the depressed ATPase activity may be Mg^{+2} control of the myofibril protein system. At the cellular level, muscle contraction is initiated by Ca^{+2} binding to the low affinity (Ca^{+2} specific) binding site of Troponin C (Tn-C). Through interaction with the other regulatory proteins this Ca^{+2} binding results in a change in the structure of the actin-tropomyosin containing thin filament that allows actin myosin interaction to take place (Mannherz and Goody, 1976). The degree of actin myosin interaction is often measured by the activity of the myofibril ATPase enzyme which catalyzes the hydrolysis of ATP allowing the actin filament to slide over the myosin filament (Huxley, 1972). Several studies (Kerrick and Donaldson, 1975; Solaro and Shiner, 1976) have shown that as the free Mg^{+2} concentration is increased the Ca^{+2} concentration required to activate tension development or myofibrillar ATPase is increased. Cardiac Tn-C contains three binding sites, two which have a high affinity for Ca^{+2} (K_{Ca} $5x10^4$ M^{-1}) and also bind Mg^{+2} competitively (K_{Mg} $5x10^4$ M^{-1}) termed the Ca^{+2}-Mg^{+2} sites and one regulatory site with a low affinity for Ca^{+2} called Ca^{+2} specific sites (K_{Ca} $5x10^6$ M^{-1}). Because Mg^{+2} does not bind directly to the low affinity Ca^{+2} specific sites even at high concentrations, it has been determined that increased Mg^{+2}

concentrations may affect the affinity of the Ca^{+2} sites for Ca^{+2} rather than a direct competition between the two ions (Potter et al, 1981). Since electron micrographs of myofibrils from diabetic hearts revealled that there is less thin filament material (Giacomelli and Weiner, 1979), it is of interest to observe whether the changes in the contractile properties of the diabetic heart are related to a change in the biochemical indices of cardiac muscle contraction namely Mg^{+2} regulation of the myofibril protein complex.

Myofibril ATPase activity is adaptable to demands placed upon the heart. For example, cardiac myofibril (Baldwin et al, 1977; Rupp, 1980; Hearne and Gollnick, 1961), myosin (Bhan and Scheuer, 1975, Penparkgul et al, 1980a; Medugorac, 1975; Wilkerson and Evonuk, 1971), and actomyosin (Guisti et al, 1978; Penparkgul et al, 1980a; Bhan and Scheuer, 1972; Malhotra et al, 1976) ATPase activity have been enhanced with training programs. These biochemical changes are associated with physiological improvements in ventricular function as measured by increases in cardiac output (Penpargkul and Scheuer, 1970), Vmax (Bhan and Scheuer, 1972), and max dp/dt (Schaible and Scheuer, 1979), negative dp/dt (Bersohn and Scheuer, 1977), and tension output (Tibbits et al, 1978).

The cellular adaptation of cardiac muscle to exercise seems to be related to the type and amount of exercise performed. Both moderate and intense swim programs lead to significant (17-56%) increases in ATPase activity while only the very intense bouts of treadmill exercise show very minimal increases in the cardiac ATPase activity (Baldwin et al, 1977; Resink et al, 1981; Penpargkul et al, 1980a), indicating that distinct differences may be apparent concerning the stress that running and swimming place on the heart.

Since the cardiac myofibril ATPase activity is adaptable to demands placed on the heart, the purpose of this investigation was to determine if the cardiomyopathy observed in chemically induced diabetes associated with depressed ATPase activity can be ameliorated with physical training, and to observe the effects of Mg^{+2} on the activation of Ca^{+2} and Mg^{+2} dependent myofibril ATPase activity from the hearts of diabetic and swim trained animals.

METHODOLOGY

Animal Care

A total of thirty four male Wistar rats (200g) were obtained from Woodlyn Farms Ltd., Guelph Ontario. The animals were housed in individual cages and fed a diet of Purina Laboratory Chow and water ad libitum. They were subjected to a controlled environment (temperature 20± 1° C; humidity 35± 3%) and maintained on a reverse day night cycle of twelve hours with the dark period lasting from 8 a.m. to 8 p.m. All training and laboratory activity took place during this time.

Experimental Design

The animals were randomly assigned to one of five groups; sedentary control (SC), sedentary diabetic (SD), training control (TC), diabetic training (DT), and a fourteen day diabetic control group. The training program for the exercise groups consisted of a progressive swim program four days per week, in water maintained at a temperature of 33± 1°C. To increase the intensity of the training session, lead weights equal to three percent of their body weight were attached to their tails. The animals were familiarized with the program for fifteen and twenty minutes on day one and day two respectively with the swim time progressively increased to a final swim time of ninety minutes by the eighth and final week (Table I).

Diabetes was induced by streptozotocin (60 mg/kg) prepared in a citrate buffer (ph 4.5) injected intravenously into the penal vein of ether anesthesized fasted rats. The control animals were injected with an equal volume of citrate buffer. Diabetes was assessed one, four, and eight weeks following the injection by collecting 5 ul of blood from the tail vein into a heparinized capillary tube and performing plasma glucose

determinations in duplicate on the YSI Glucose Analyser. In additon, urine glucose values were obtained at four and eight week intervals. Dilution factors (distilled water) for the plasma and urine glucose determinations were 6 and 21 times the volume of blood utilized (1.0 ul). Criteria for diabetes was a glucose concentration above 450 mg%.

Tissue Sampling

Forty eight hours following the final training session the animals were sacrificed by decapitation, exsanguinated and the cardiac muscle excised (Belcastro et al, 1983). Once removed, the heart was rinsed in cold saline, both ventricles were dissected from the remainder of the heart and trimmed of all visible fat and related tissue. The ventricular mass was weighed, frozen in isopentane, cooled by liquid nitrogen, and stored at -70°C for determination myofibril ATPase and Calcium binding activity.

Myofibril Isolation

The cardiac tissue was homogenized with a Polytron Pt-10 for twenty seconds in twenty volumes of 0.039 M Sodium Borate, 25 mM Potassium Chloride, 5 mM Sodium Ethylenediamine Tetracetate (EDTA) and centrifuged at 1000 xg for ten minutes. The supernatant was discarded and the muscle pellet resuspended and recentrifuged in the same solution. Following centrifugation the 1000 xg pellet was resuspended with a Borate-Tris buffer and prepared for ATPase determination by washing the pellet twice. Following resuspension, small (0.1 ml) aliquots were taken for protein determination in quadruplicate (Lowry et al, 1951) and the myofibril fraction adjusted to two milligrams of protein per milliliter.

Myofibril ATPase Determination

Myofibril ATPase activity was carried out as described by Goodno et al (1978). The reaction mixture contained 100 mM KCL, 4 mM Tris, 0.04, 1.0, or 10.0 mM $MgCl_2$, 10 uM free Ca^{+2}, and 0.5 mg/ml of myofibril protein. The reaction was started with the rapid addition of 5 mM MgATP to the sample, mixed thoroughly, and allowed to incubate for 5 minutes at 30°C. The reaction was stopped by the addition of 20% cold TCA. The samples were centrifuged at 1000 xg for ten minutes to precipitate the protein. The supernatant was used for determination of inorganic phosphate (P_i) carried out in quadruplicate by the method of Taussky and Shorr (1953), and expressed as (umol $P_i^{-1} \cdot mg^{-1} \cdot min^{-1}$).

Calcium Binding

Ca^{+2} binding by the myofibril protein was carried out by the modified method of Solaro and Shiner, (1976). A 3 ml volume consisting of working solution (Na_2ATP, KCl, $MgCl_2$, NaCl, pH 7.0), suspension medium, Ca^{+2} binding solution (5uM free Ca^{+2}, EGTA, imidazole, pH 7.0), and Ca^{45} (1.25 uCi) was mixed and preincubated for three minutes at 30°C. The reaction was stopped by filtering the solution through a Millipore HAWP - 0.45 micron filter. Small aliquots (0.1 ul) of the filtrate was utilized for final Ca^{+2} binding determination.

Statistical Design

Mg^{+2} ATPase activity (umol $P_i \cdot mg^{-1} \cdot min^{-1}$) of SC, SD, TC, and DT groups incubated in either 10 um Ca^{+2}-EGTA or EGTA at varying free Mg^{+2} concentrations was anaylzed with a three way analysis of variance (2x2x3) . Any statistic displaying a significant F value

was further tested through a one way ANOVA. Significance among F values was determined through the Student- Newman-Keuls post hoc test.

One-tailed t tests were used to determine significant differences between the groups for Mg^{+2} ATPase activity with the varying Mg^{+2} concentrations. Significant differences were determined at a 95% confidence interval ($p > 0.05$).

Table I: Training Schedule of Endurance Trained Rats (in minutes, 3% BW attached to tails)

Week	Mon.	Tues.	Wed.	Thurs.	Fri.	Sat.	Sun.
1	15	20	Rest	25	30	Rest	Rest
2	30	35	Rest	40	45	Rest	Rest
3	50	55	Rest	60	65	Rest	Rest
4	65	65	Rest	70	70	Rest	Rest
5	70	70	Rest	75	75	Rest	Rest
6	70	70	Rest	75	75	Rest	Rest
7	75	80	Rest	75	75	Rest	Rest
8	85	85	Rest	90	90	Rest	Rest

LIMITATIONS OF THE STUDY

1) This study utilized animals with uncontrolled diabetes as opposed to diabetes controlled by daily insulin injections. Since most forms of human diabetes are now controlled via insulin injections it would be beneficial to to add a sixth group to the study with controlled diabetes.
2) Male Wistar Rats were utilized in this study as opposed to human subjects. It is difficult to extrapolate the results gained from animal studies to man due to differences between the two species in metabolism, circulatory parameters, and exercise patterns.
3) Strain differences and the origin of the strain must be taken into consideration since some strains are more suceptable to the diabetogenic effects of streptozotocin (Ganda et al, 1976).

DEFINITIONS

DIABETES MELLITUS A familial constitutional disorder of carbohydrate metabolism characterized by inadequate secretion or utilization of insulin by polyuria and excessive amounts of sugar in the blood and urine, and by thirst hunger and loss of weight.

DIABETES INSIPIDUS A disorder of the pituitary gland characterized by intense thirst and by the excretion of large amounts of urine.

STRESS The response of the body to a particular stimulus.

UNCONTROLLED DIABETES The diabetic condition that is not controlled by medication.

CONTROLLED DIABETES The diabetic condition that is controlled by medication.

ACCURACY Degree of conformity of a measure to a standard or true value.

RESULTS

Heart and Body Weight

Heart weight and body weight values for the experimental groups are presented in Table II. The body weights and left ventricular wet weights were lower in diabetic than in control animals with the heart weight to body weight ratios (HW/BW) substantially higher in the diabetic group ($p < 0.05$). At the end of eight weeks of swimming the body weights were greater in the sedentary than the conditioned rats. Heart weights did not differ between the two groups and the HW/BW ratios were higher in the conditioned groups ($p < 0.05$). No changes in these parameters were apparent between the diabetic sedentary and diabetic trained groups.

Plasma and Urine Glucose

Plasma and urine glucose values are shown in Table II for the SC, DT, and SD groups. Samples were not taken from the TC group since all control animals have approximately the same urine glucose values regardless of training. Blood glucose values rose to diabetic values within two weeks post injection and remained stable for the duration of the experiment ($p < 0.05$). At the end of the experimental period blood and urine glucose values were approximately six and nineteen times higher than control values respectively. At the completion of the study there were no significant differences between the diabetic sedentary and diabetic trained groups.

Effects of Diabetes on Myofibril ATPase Activity

Cardiac myofibril ATPase activity was depressed ($p < 0.05$) in preparations from hearts of diabetic animals at the three Mg^{+2}

concentrations in the presence of 10 uM Ca^{+2} (Figure I). The cardiac myofibril ATPase activity was reduced by 45, 45, and 50% at 0.04, 1.0, and 10.0 mM Mg^{+2} in diabetic hearts compared to controls ($p< 0.05$). There was a significant reduction in myofibril ATPase activity for both control and diabetic animals when the Mg^{+2} concentration was increased from 0.04 to 10.0 mM Mg^{+2} with the differences greater for the diabetic (25%) compared to the control (16%) animals ($p< 0.05$). The Mg^{+2} stimulated ATPase activity (independent of Ca^{+2}) was similarly depressed in diabetic animals at all Mg^{+2} concentrations ($p< 0.05$). Increasing Mg^{+2} concentrations (0.04 - 10.0 mM) resulted in an elevated ATPase activity for normals from 0.034 $\pm$ 0.004 to 0.042 $\pm$ 0.004 umol inorganic phosphate(P_i).mg^{-1}·min^{-1}. For diabetics comparable activities were 0.021 $\pm$ 0.005 to 0.020 $\pm$ 0.004 umol P_i·mg^{-1}·min^{-1}. (Figure II).

The Effect of Swim Training on ATPase Activity

Myofibril ATPase activity was increased by 25% in trained hearts at 0.04, 1.0, and 10.0 mM Mg^{+2} when compared to control hearts ($p< 0.05$) (Figure III). There was a significant decrease in myofibril ATPase activity in the sedentary and conditioned hearts when Mg^{+2} concentration was increased from 0.04 to 10 mM Mg^{+2} ($p< 0.05$) with the differences being slightly greater for the trained animals (21%) compared to the controls (18%). Conversely, there was no change in Mg^{+2} stimulated ATPase activity between trained and control animals nor was there any difference when Mg^{+2} concentration was increased from 0.04 to 10 mM Mg^{+2}.

The Effect of Swim Training on ATPase Activity in Diabetic Animals

Myofibril ATPase activity was significantly depressed in diabetic trained animals at 0.04 and 1.0 mM Mg^{+2} with no apparent change at 10 mM Mg^{+2} ($p < 0.05$) (Figure III). As Mg^{+2} concentration increased from 0.04 and 1.0 mM to 10mM Mg^{+2} a significant depression of myofibril ATPase activity was observed in the diabetic trained and diabetic sedentary hearts. The Mg^{+2} stimulated ATPase activity decreased by 12.5% in the diabetic training group at 0.04 and 1.0 mM Mg^{+2} with no changes found at 10 mM Mg^{+2}. Increasing Mg^{+2} concentration from 0.04 to 10mM Mg^{+2} did not affect the ATPase activity in either group.

Calcium Binding

There were no significant changes in Ca^{+2} binding between the four groups at 0.04 or 1.0 mM Mg^{+2} although Ca^{+2} binding values for the training diabetic and training control groups were slightly but not significantly higher than their control group counterparts. (Table III).

Table II: General Features of the Experimental Groups

		SC	TC	SD	DT
Body Wt. (gr)		468 ± 22*°	406 ± 7	300.±16	287±20
Heart Wt. (gr)		1.15 ± 0.05*	1.14 ± 0.036	0.87 ± 0.046	0.819±0.057*
HW/BW		2.5 ± 0.09*°	2.8 ± 0.04	2.9 ± 0.144	2.9 ± 0.08
Plasma Glu.	1	126 ± 3	-----	910 ± 76●	748 ± 32.
(mg/dl)	4	126 ± 3	-----	687 ± 38	742 ± 73
	8	124 ± 4	-----	758 ± 43	688 ± 40
Urine Glu. (mg/dl)	8	360 ± 137	-----	6805 ± 923	6886 ± 922

° = SC vs TC

* = SC vs SD

● = SD vs DT

Table III: Calcium Binding Data

Group	0.04 mM Mg	Range	1.0 mM Mg	Range
TC	2.98	2.7-3.3	2.31	2.2-2.4
SC	2.43	1.9-2.9	2.10	2.0-2.4
DT	2.80	2.4-2.7	2.80	2.3-3.4
SD	2.20	2.2-2.5	2.40	2.1-2.6

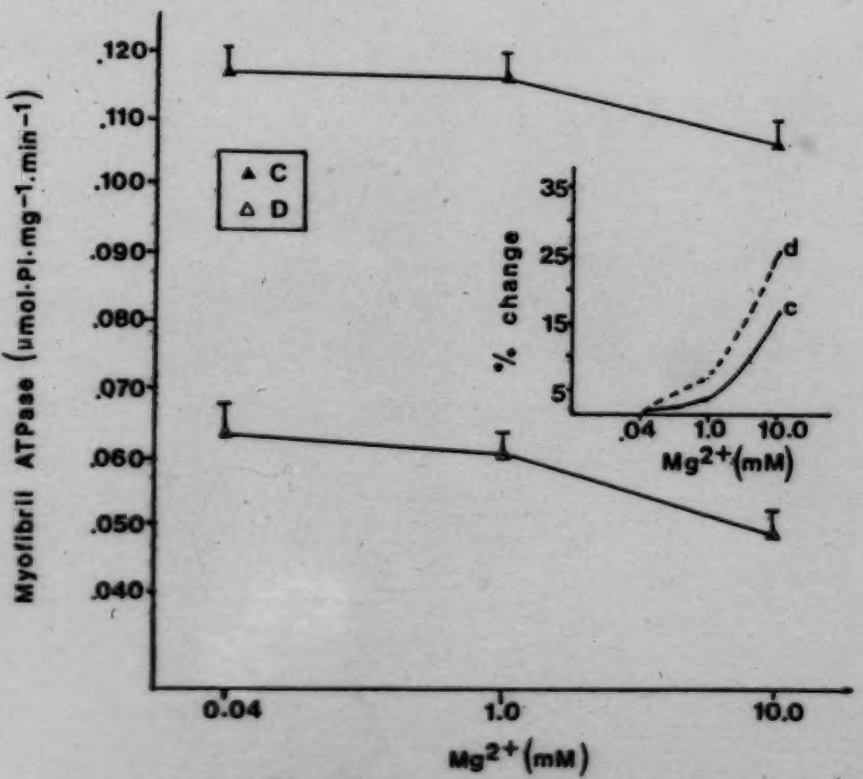

Figure I: Ca^{2+}-Mg^{2+} Myofibril ATPase with Diabetes

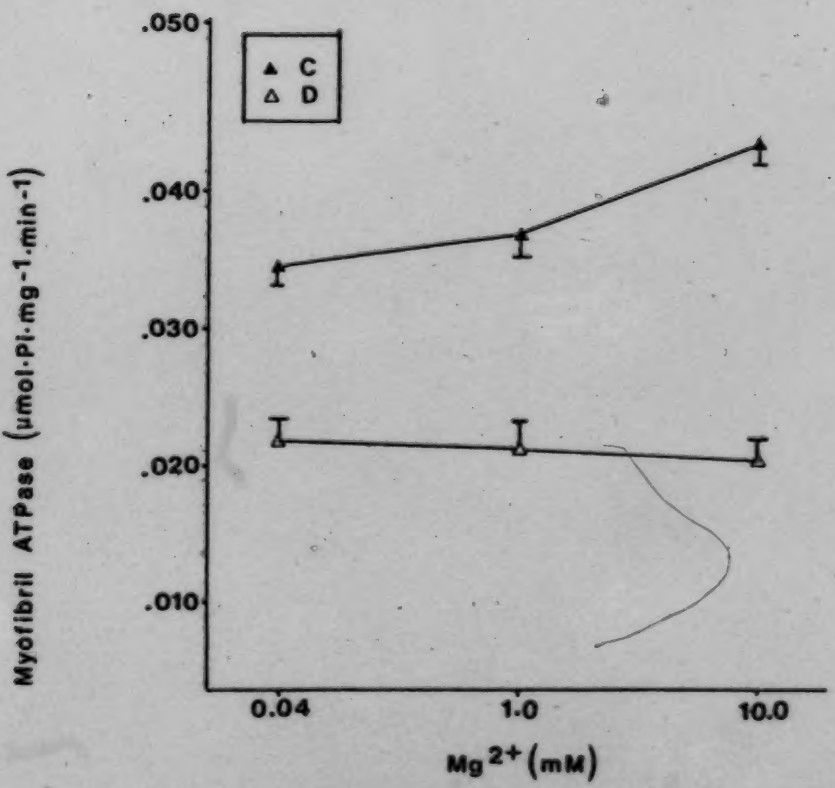

Figure II: Mg^{2+}-ATPase of Control and Diabetic Cardiac Muscle

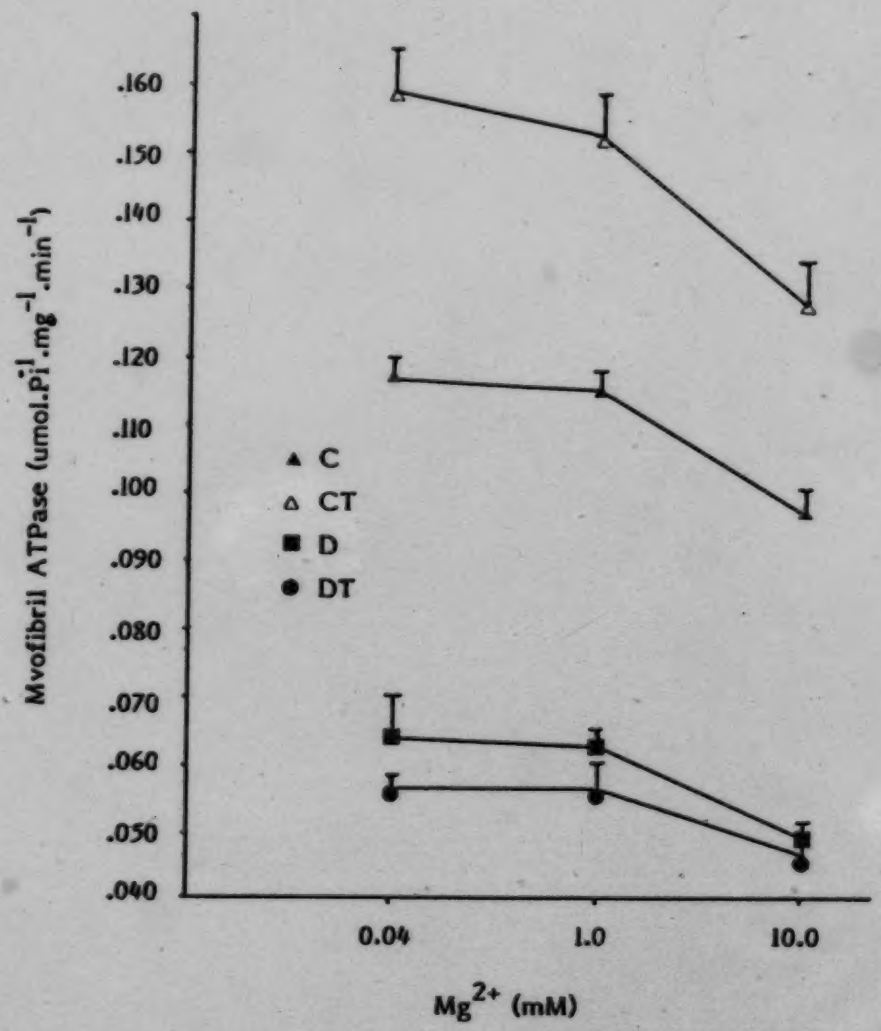

Figure III: $Ca^{2+}Mg$ Stimulated ATPase activity in Control, Diabetic and Trained Hearts

DISCUSSION

The lower heart weight (HW), body weight (BW), and higher heart weight to body weight ratios (HW/BW) observed in the diabetic animals is in general agreement with other studies (Penpargkul et al, 1980b; Pierce and Dhalla, 1981; Malhotra et al, 1981). With regard to swimming, the decrease in BW, and increased HW/BW ratio, with no change in HW is in agreement with other swim training studies (Bhan and Scheuer, 1972; Malhotra et al, 1976; Penpargkul and Scheuer, 1970; Bersohn and Scheuer, 1977; Oscai et al, 1971a). The decreased body weight of the conditioned animals responsible for the increased HW/BW ratio observed in this study is probably due to the decreased food consumption usually observed throughout the course of an exercise program (Nance et al, 1977). Blood glucose values reached diabetic levels (682$\pm$97 mg/dl) within fourteen days. Previous studies have shown that following the injection of streptozotocin blood glucose values rose to greater that 400 mg/100ml within twenty four hours (Malhotra et al, 1981; Penpargkul et al 1980b). The plasma glucose values (758 - 910 mg/dl) were higher when compared to other studies (Pierce and Dhalla, 1981; Penpargkul et al, 1980b; Penpargkul et al, 1981; Malhotra et al 1981), possibly due to the different injection sites and/or animal species utilized. It may be possible that a greater percentage of streptozotocin is absorbed through the penal vein when compared to the tail vein injections, however the extent of diabetes as measured by blood glucose does not appear to be related to the degree of cardiomyopathy (r=0.28) (Belcastro and McLean, 1983).

In the present investigation when severe diabetes (greater than 600 mg/dl) was induced by the intravenous injection of streptozotocin, profound alterations occurred in the enzymatic activity of the cardiac contractile proteins. This was evidenced by a depressed myofibril $Ca^{+2}Mg^{+2}$ ATPase activity at the three Mg^{+2} concentrations investigated which is consistent with a previous report (Pierce and Dhalla, 1981). In addition, the myosin ATPase activity (Dillman, 1980) and actomysoin ATPase activity (Malhotra et al, 1981) in the hearts from diabetic animals have been reported to be depressed. Dillman (1980) and Malhotra et al, (1981) have determined that this may be due to a redistribution in the pattern of myosin isoenzymes V_1 V_2 and V_3. The predominating V_1 isoenzyme in control tissue was replaced by the V_3 isoenzyme in diabetic tissue which has a ten fold lower ATPase activity that V_1. The new expression of an isoenzyme of myosin has been suggested to be responsible for altered ATPase activities in various states (Hoh et al, 1977). Pierce and Dhalla, (1981) found no difference in the dependence of myofibrillar ATPase activity on free Ca^{+2} concentration in the diabetic heart while our results indicated no change in Ca^{+2} binding to the myofibril. Pierce and Dhalla (1981) using varying KCl concentrations determined that structurally different forms of the protein may be found in diabetic preperations. In addition to these subtle structural alterations based on ethylene glycol studies they suggest conformational changes at or near the active site of ATPase activity through sulphydryl group modification may inactivate myosin ATPase activity. The possibility must be considered that the depression was caused directly by the effect of streptozotocin on the heart rather

than the diabetic effect of the drug. Malhotra et al, (1981) examined this question by administrating 3-0 methyl glucose, a sugar when administered before the streptozotocin prevents its diabetogenic effect, (Ganda, et al, 1976). Preparations from the hearts of these animals in which diabetes had been blocked displayed no depression in actomyosin ATPase activity, indicating that streptozotocin injection alone is not sufficient to cause the deliterous effects on the heart. Diabetes must develop for these abnormalities to occur. It should be noted that when insulin is re-administered the cardiomyopathic effects of the diabetes is retarded (Belcastro and McClean, 1983).

Swim training resulted in an enhanced (36%) myofibril Ca^{+2}-Mg^{+2} ATPase activity. These observations are in general agreement with the data from swimming studies (Hearne and Gollnick, 1961; Rupp, 1981; Bhan and Scheuer, 1972; Penpargkul et al, 1980a; Wilkerson and Evonuk, 1971, Medugorac, 1975; Gusti et al, 1978; Malhotra et al, 1976) but are in contrast to those of running studies where no change (Dowell et al, 1977; Tibbits et al, 1978; Baldwin et al, 1975; Penpargkul et al, 1980a) or minimal changes occur (Baldwin et al, 1977; Resink et al, 1981; Penpargkul et al, 1980b).

The intensity and duration of the exercise program employed corresponds to the degree of ATPase changes incurred. Time studies have demonstrated that during the first eight weeks of the program the ATPase activity increases in proportion to the duration and severity of the program (Bhan and Scheuer, 1972; Wilkerson and Evonuk, 1971).

Therefore the different intensity and duration employed from one study to another may account for some of the discrepency observed between studies, although the most important factor concerning the differences in ATPase activity appears to be the type of exercise program utilized. Both moderate and intense swimming programs lead to significant increases in ATPase activity while only the very intense bouts of treadmill exercise show slight increases in ATPase activity of the contractile proteins (Baldwin et al, 1977; Resink et al, 1981; Penpargkul et al, 1980a). Water immersion itself considered a possible explanation was eliminated by Penpargkul et al (1980a) who found no change in ATPase activity. This has led to the speculation that very distinctive differences are apparent concerning the stresses that swimming and running place on the heart although the reasons for the biochemical differences remain to be explained.

The factors responsible for the altered ATPase changes in cardiac tissue with training are uncertain. Rupp, (1981) has detected an isoenzyme pattern alteration where hearts from conditioned animals show a single band of the isoenzyme V_1 which displays the highest ATPase activity of the three isoenzymes V_1, V_2, and V_3 found in control hearts. In addition, Medugorac (1975), has found an increased amount of light chain 1 (LC 1) in swim trained rats, while Resink et al (1980) has found a greater concentration and incorporation of phosphate into LC2 from running trained animals. Concomitant with the myofibril, myosin, and actomyosin changes are corresponding

alterations in the contractility of the heart which include an increase in max dp/dt (Penpargkul and Scheuer, 1970), and tension development (Schaible and Scheuer, 1979) along with a faster rate of relaxation (Bershon and Scheuer, 1977). Thus it has been postulated that the reported exercise induced increase in cardiac contractile protein ATPase activity following training represents a cellular mechanism to account for the improvement in cardiac contractile function.

The swim training program did not enhance the myofibril Ca^{+2} Mg^{+2} ATPase activity of the diabetic animals in contrast to the results for the non diabetic group. Moreover, the myofibril ATPase activities for the trained diabetic animals were significantly reduced by 13% at 0.04 and 1.0 mM Mg^{+2} respectively. Ca^{+2} binding was similar between the two groups and therefore cannot account for the differences between diabetic trained and diabetic control animals. Because diabetic cardiac tissue utilizes lipid stores rather than glucose, in hearts not working at maximal loads the energy production from the oxidation of lipids compensates for the reduced glucose metabolized in the diabetic state which allows the diabetic heart to maintain a normal function (Neely and Morgan, 1981). Under maximal stress an intrinsic defect of the heart to produce ATP seems possible (Sinclair- Smith, 1979), which may be consequent to the increased tissue levels of triglycerides, free fatty acids, long chain acetyl Co A, and citrate. Evidence has recently been presented that an accumulatation of these substances interfere with the efficiency of the myocardium. Furthermore, Opie (1977) has demonstrated that

promotion of lipid uptake by the heart with regional ischemia tends to exaggerate the extent of ischaemic injury. Since physical exercise is considered to be a stress it may be possible that the biochemical alterations detected in the diabetic heart may have been exaggerated when additional stresses like swimming and the fear of drowning were placed on the animal.

A second possibility for the further decrease in ATPase activity in the diabetic animal following a training program may be related to protein degradation. It has been determined that normal growth requires insulin, and in the diabetic organism protein synthesis and amino acid accumalation in muscle are severely reduced (Cahill et al, 1969). In non diabetic rat soleus or diaphram muscle exercise reduces the rate of protein degradation in muscle (Goldberg et al, 1974; 1975), along with a decrease in the plasma insulin levels (Terjung, 1979). It is possible that the decreased insulin levels in non diabetic tissue has no effect on the protein degradation process while the decreased insulin levels during exercise together with the decreased insulin levels in diabetes act synergystically in a negative manner leading to an even greater degree of protein degradation concomitant with the decreased ATPase activity.

Mg^{+2} Regulation

Increasing the Mg^{+2} concentration from 0.04 to 10.0 mM of Mg^{+2} resulted in a depressed ATPase activity in the four groups studied, in agreement with Solaro and Shiner (1976) who have shown that as free Mg^{+2} concentration is increased the Ca^{+2} concentration required to activate tension development or myofibril ATPase activity is increased. It has been suggested that Mg^{+2}

might bind competitively to the low affinity Ca^{+2} specific sites at high Mg^{+2} concentrations (Rupp, 1980). Potter et al (1981) have determined that even at high Mg^{+2} concentrations the Mg^{+2} ion cannot bind to the low affinity site. It has therefore been assumed that the increased Mg^{+2} concentration must reduce the affinity of the low affinity sites for Ca^{+2} rather than directly competing with the Ca^{+2} ion for a position on the site.

The decrease in myofibril ATPase activity from 0.04 to 10.0 mM Mg^{+2} is greater in the diabetic hearts (25%) than the control hearts (16%) with minimal differences apparent between the conditioned and control animals. It can then be concluded that there is an alteration in the Mg^{+2} dependency of the cardiac myofibril with diabetes, that is not observed following swim training. This alteration in Mg^{+2} dependency with diabetes appears to be partially responsible for the reduced activity of the myofibril ATPase enzyme. At present it is unclear as to the mechanism of the altered Mg^{+2} regulation, however it may be related to the binding affinity of Mg^{+2} to the high affinity Ca^{+2}-Mg^{+2} sites. These sites appear to function in stabilizing the Tn-C complex with most of the conformational changes induced by Ca^{+2} binding to the Tn-C complex occuring at this site, (Potter and Gergley, 1975). It may be possible that Mg^{+2} binds to these sites exclusively in the presence of high Mg^{+2} concentrations, and induces a different conformational change than Ca^{+2} that could affect one of the regulatory events in the contraction process.

Examination of the basal ATPase activity (Mg^{+2} ATPase) reveals no differences between the training and control and the

diabetic and control hearts indicating that the Mg^{+2} effects are not due to higher basal ATPase activity. This implies that something related to the contractile process itself must be causing the Mg^{+2} effects observed in the diabetic animals. Since Giacomelli and Weiner (1979) have determined that the myofibrils contain less thin filament the changes in Mg^{+2} dependency and ATPase activity associated with diabetes may be related to structural changes in the thin filament.

In summary this study demonstrates that the depressed myofibril ATPase activity of diabetic hearts is a function of Mg^{+2} regulation, while the increased ATPase activity in the conditioned animals is not a function of Mg^{+2} regulation. In addition, the depressed myofibril ATPase activity in the diabetic animals is not ameliorated with physical training.

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Addendum

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APPENDIX A

REVIEW OF LITERATURE

STRUCTURE

The cytology of cardiac muscle has been extensively investigated with several correlations between structure and function becoming increasingly evident. Of primary physiological importance is the conversion of chemical energy into mechanical work; the energy is provided in the form of ATP, and the work is manifest by the sliding filaments located in the cytoplasm of muscle cells. The trigger for contraction is an influx of Ca^{+2} ions into the matrix, while subsequent relaxation of the cells requires the uptake and sequestration of Ca^{+2} by the sarcoplasmic reticulum (SR). The structures that are important in these processes will be the focal point of the following discussion.

CARDIAC ULTRASTRUCTURE

The cells of the working myocardium contain large numbers of myofibrils and mitochondria enclosed within the sarcolemma which delimits the cell contents. These cells contain two distinct intracellular membrane systems: the transverse tubular system (T-system) and the SR. Together these structures occupy less than 90% of the cell volume, the remainder consisting mainly of cytosol and nuclei (Katz, 1977).

The myocardial cell is enclosed by the sarcolemma, a 7.5-9.0 nm unit plasma membrane which defines the boundary between the intra and extracellular spaces. The sarcolemma in both cardiac and skeletal muscle is composed of a trilaminar unit membrane. The

bilayered plasma membrane (sarcolemma) is the basic cell unit membrane surrounded by a two layered glycocalyx or basement membrane (50 nm) which lies on the exterior of the sarcolemma. One layer termed the surface coat (20 nm thick) is an extension of the plasma membrane, while the second more peripheral layer, the external lamina (30 nm thick) extends from the surface coat (Langer, 1978; Adams and Schwartz, 1980). The plasma membrane appears to function as a selective barrier to the free flow of low molecular weight substances and ions such as sodium, potassium, and calcium , while the negatively charged glycocalyx may be the site of Ca^{+2} binding and exchange across the cell membrane in cardiac muscle (Adams and Schwartz, 1980).

At many sites on the surface of cardiac muscle cells the sarcolemma is deeply invaginated in the form of long slender tubules called individually a transverse tubule or t-tubule and collectively the T-system (Sommer and Johnson, 1978). Several prominent differences exist between skeletal and cardiac muscle in the geometry the T-system. Skeletal t-tubules do not appear to contain the glycoprotein surface coat of the sarcolemma. The T-system is much larger in diameter and more variable in size in cardiac muscle than skeletal muscle. In mammalian skeletal muscle two transverse tubules are present at the level of sarcomere situated at the boundary of the A and I bands while in cardiac muscle the transverse tubules are generally associated with the Z bands, thus there is only one t-tubule per sarcomere. Finally, in contrast to skeletal muscle the glycoprotein surface coat material of cardiac peripheral sarcolemma continues down into the t-tubules and therefore is brought into proximity with the contractile filaments

(Sommer and Johnson, 1978; Adams and Schwartz, 1980).

The SR consists of a network of intracellular tubules that surround the bundles of contractile proteins, forming specialized structures that are in contact with the sarcolemma and the T-system. Here the tubules of the SR flatten to form subsarcolemmal cisternae. The composite structure formed by the subsarcolemmal cisternae and the adjacent region of either the sarcolemma or t-tubule is called the dyad (Katz, 1977), the region within the membrane where Ca^{+2} is stored (Podolsky, 1975). Unlike the SR in skeletal muscle which is arranged in a parallel fashion anastmosing freely in the region of the A band, cardiac SR has a somewhat random orientation, connecting at virtually all levels of the sarcomere (Adams and Schwartz, 1980).

The function of the SR as a primary regulator of myoplasmic Ca^{+2} during the contraction relaxation cycle is well established for striated muscle (Inesi and Malan, 1976). It is thought that cardiac SR like skeletal SR acts as the primary site for Ca^{+2} release to the myoplasm during excitation to initiate contraction, and Ca^{+2} uptake from the myoplasm to initiate relaxation.

In contrast to skeletal muscle, it appears that some Ca^{+2} crosses the surface membrane of the cardiac cells during the action potential whereas very little or no Ca^{+2} crosses the surface membrane of skeletal muscle cells (Rich and Langer, 1975). A Ca^{+2} induced release of Ca^{+2} has been proposed whereby a small amount of Ca^{+2} crosses the sarcolemma during the action potential, insufficient by itself to activate the myofilaments, induces the release of Ca^{+2} from the SR sufficient for activation (Fabiato and Fabiato, 1977).

Morphology of the Myofibril

The myofibrils which constitute about 20% of the volume of cardiac muscle (Katz, 1977) are arranged in regular arrays of thick and thin myofilaments enclosed within a sarcolemma. The fibers are connected to one another end to end by tight junctions known as intercalcatred discs which are densely staining transverse bands that characteristically appear at right angles to the long axis of cardiac myofibers (Katz, 1977). Unlike skeletal muscle cells which are long parallel uniform fibers, cardiac fibers bifurcate and connect with adjacent fibers to form a three dimensional network (Adams and Schwartz, 1980).

The contractile material along the long axis of the cardiac muscle is alternately striped by a dark anisotropic (A) band and a light isotropic (I) band created by the interdigitation of 2 sets of myofilaments; the thick myosin filaments and the thinner actin filaments. The I band, composed of actin is bisected by a narrow darkly staining Z line which consists mainly of a protein constituent called alpha actinin (Masaki et al, 1967; Stromer and Goll, 1972; Ebashi et al, 1966). Recent studies indicate that alpha actinin connects F actin filaments to the Z line in both cardiac and skeletal muscle (Toyo-Oka and Masaki, 1979). Proper orientation is maintained by the separation and looping of the protein which composes the backbone of thin filaments through the Z line returning to the center of the sarcomere in one of the adjacent thin filaments (Katz, 1977). The actin filaments insert at one end of the Z line, extend through the I band and one half of the A band terminating on either end of the H

zone, the central region within the A band. A broad dense M band is found in the center of the H zone which consists of myosin filaments only. The myosin filaments attach to the M band and are held together by M protein which maintains the myosin filaments in a regular hexagonal array in the center of the sarcomere by cross linking the thick filaments (Mannherz and Goody, 1976). The myosin filaments which are continuous throughout the entire A band contains a hexagonal array of the thick filaments each of which is surrounded by six thin filaments which lie at trigonal points between adjacent thick filaments. The sarcomere, the fundamental morphological unit of striated muscle is defined as the region between the two Z lines, and thus consists of a single A band plus two adjacent half I bands (Mannherz and Goody, 1976).

Myofibrillar Fine Structure

I. Myosin

It is now well established that myosin is the chief constituent of the thick filaments of striated muscles (Katz,1970). Myosin, a hexameric rod shaped molecule (MW 500,000), (Katz, 1977) is 140 nm long and 2 nm wide terminating in two globular heads 7 nm in diameter (Morel and Pinset-Harstrom, 1975). Mild treatment with trypsin yeilds two subfragments; light meromyosin (LMM) and heavy meromyosin (HMM) with further papain digestion splitting HMM into two segments, HMM S-1 and HMM S-2 (Katz, 1970; Mannherz and Goody, 1976). The tail region consists of an insoluble enzymatically inactive molecule and a water soluble enzymatically active molecule (HMM S-2), both composed of two alpha helices twisted around one another to form a

coiled coil. HMM S-2 terminates when both polypeptide chains untwist to form two water soluble globular regions referred to as HMM S-1 (MW 140,000). H.E. Huxley (1969, 1971), using electron microscopy and X-ray diffraction tecniques envisaged HMM S-2 acting as a stiff light rod with hinges at both ends, that is HMM S-2 is connected to LMM by a hinge and is joined to HMM S-1 by a second hinge, thus enabling the globular head to swing out when attaching to the thin actin filament (Mannherz and Goody, 1976).

The S-1 region contains both the ATPase activity and the actin combining ability of cardiac and skeletal muscle myosins (Katz, 1970; Mannherz and Goody, 1976; Morel and Pinset-Harstrom, 1975). Ramirez, (1979) proposed a model for the active site of skeletal and cardiac myosin that relates directly to the ninety two amino acid fragment P10 of myosin recently described by Elzinga and Collins, (1977). In this model the MgATP substrate, an eight membered cyclic complex fits tightly into a sixteen amino acid segment of P10 and interacts with seven of its amino acids. At this site it is postulated that a rare amino acid N-methyl histidine (position 69) present in skeletal muscle and histidine (position 69) in cardiac muscle heavy chain myosin functions as the donor ligand for Mg^{+2}. Once this ligand is beside the metal, other amino acids form a pocket around MgATP. These amino acids include tyrosine 72 (attached to Mg^{+2}), histidine 76 (donates a proton to the P of ATP), Lysine 78 (binds electrostatically to P_B of ATP) Aspartate 66 (forms a hydrogen bond to the 6-amino group of adenine) and phenylalanines 80 and 81 which flank the ring of ATP. (See Ramirez, 1979 for the rationale behind the model).

Also located on the globular head are a number of low molecular weight components termed light chains which appear to be involved in the hydrolytic activity of myosin (Mannherz and Goody, 1976). There appears to be three types of light chains in skeletal muscle; an 18,000 D light chain which can be removed from myosin by reacting with 5 5' dithiobis (2 nitrobenzoic acid) termed the DTNB light chain (LC2) containing two thiol groups in identified sequences (2 mol/mol myosin) and two single thiol sequenced light chains MW 16,000 (LC3) and 25,000 (LC1) dissociated from the molecule at ph 11 termed "alkali light chains" (2 mol/mol myosin), (Lowey and Risby, 1971; Weeds and Pope, 1971; Weeds and Lowey, 1971). The DTNB chains can be removed without loss of ATPase activity while loss of ATPase activity ensues when alkali light chains are removed (Weeds and Pope, 1971; Weeds and Lowey, 1971).

While fast skeletal muscle appears to have three types of light chains cardiac and slow muscle contain two classes of myosin light chains; LC1 (2.05 mol/mol myosin) and LC2 (2.95 mol/mol) as measured by SDS-Page Electrophoresis (Frearson and Perry, 1975,; Lowey and Risby, 1971; Weeds and Pope, 1971; Sarker et al, 1971; Wilkman-Coffelt et al, 1973 a,b). Both atrial and ventricular myosins contain these two light chains although they differ slightly in weight, size, and charge.

The 18,000 D component of fast muscle (DTNB) and the LC2 (18 - 19,000 D) subunit from cardiac and slow skeletal muscle show a considerable degree of amino acid homology (Jakes et al, 1976; Leger and Elzinga, 1977; Frearson and Perry, 1975), although the latter two cannot be separated from the myosin molecule with DTNB (Mannherz and

Goody, 1976). It appears that this 18,000 D LC from the three muscle types can undergo phosphorylation and dephosphorylation (1 site/molecule). Recent studies indicate a specific myosin light chain kinase which catalyses the transfer of the y phosphate of ATP to a single serine residue in position 14 or 15 (Perrie et al, 1973; Collins, 1976) on the 18 - 19,000 D component (Perry, 1973; Pires et al, 1974; Frearson and Perry, 1975; Morgan et al, 1976; Perrie et al, 1973). A protein phosphotase which can remove this group is also present in the muscle (Perry et al, 1975). This phosphorylation of LC2 apparently causes an increase in actin activated myosin activities (Gorecka et al, 1976; Small and Sobezek, 1977) while removal of LC2 can affect the basic Mg^{+2} ATPase and actin activated Mg^{+2} ATPase activities resulting in enhanced actin activated ATPase activities (Malhotra et al, 1969).

Sequence similarities have also been demonstrated between cardiac LC1 and the alkali labile light chains of fast muscle although cardiac LC1 contains one additional thiol sequence not found in fast muscle (Mannherz and Goody, 1976). Removal of these LC cause total loss of Ca^{+2} ATPase activity while removal of LC2 has no effect on this parameter (Malhotra et al, 1969).

Low molecular weight component light chains g_1, g_2, and g_3 (1:2:1) have also been isolated from both skeletal (Yagi et al, 1975) and cardiac muscle myosin (Kuwayama and Yagi, 1977). The functions of g_1 and g_3 are unknown although separation of g_1 and g_3 from cardiac myosin is always accompanied by a loss of ATPase activity. The S-1 can contain either g_1 or g_3 suggesting that they are homologous proteins which may be located at analagous sites of

different myosin isoenzymes (Okamoto and Yagi, 1976).

Most research to date has concentrated on the g_2 light chain, a 5,000 D Ca^{+2} binding component of skeletal and cardiac myosin (Morimoto and Harrington, 1974; Kuwayama and Yagi, 1977). There is a difference in size of the heavy chain components of the two S-1 subfragments by about 5,000 D, the smaller S-1 cannot bind the g_2 light chain while the larger (f) component can. A 5,000 D polypeptide termed "C polypeptide" because it is located on the C terminal side of the myosin filament is necessary to bind g_2 near the link between the head and tail of myosin (Kuwayama and Yagi, 1977). A 15,000 D component protein which appears to be a proteolytic product of g_2 and can bind Ca^{+2} as g_2 does has been seperated in cardiac muscle which contains very little g_2. The subfragment which contains neither the 15,000 D protein nor the g_2 is unable to bind Ca^{+2}.

In the heart two classes of isomyosins have been detected those of the atria and those of the ventricles (Wilkman-Coffelt and Srivastova, 1979). The molecule of atrial and ventricular myosin contain two heavy chains (200,000 D) and two pairs of dissimilar light chains in equimolar ratios, the LC1 and LC2 as described in the preceding section. (Zak et al, 1982). The light chains between atrial (A) and ventricular myosins (V) are different in both size (ALC1 27,000, VLC1 19,000, ALC2 22,000, VLC2 19,000) (Hoh et al, 1977), and charge (atrial myosin is more positively charged). Differences are also apparent between atrial and ventricular heavy chains in their primary structure. The cystine content of atrial myosin is less than ventricular myosin otherwise they are similar in

their amino acid content. One additional descrepency involves nine additional peptides in atrial myosin which are not present in ventricular myosin and eleven peptides in ventricular myosin which are not present in atrial myosin (Flink et al, 1978). These differences could account for the greater Ca^{+2} ATPase in atrial vs ventricular myosin (Hoh et al, 1977).

Gel electrophoresis (Hoh et al, 1977) and specific immunofluorescent staining demonstrate the presence of specific ventricular isoenzymes V_1, V_2, and V_3, with one two or three of these isoenzymes present depending on the species (Clark et al, 1982). Since the light chains are identical in the three isoenzymes (Hoh et al, 1977), differences in ventricular myosin must be a consequence of a number of variations in their heavy chains (Zak et al, 1982). Two distinct myosin heavy chains HC alpha (A)and HC beta (B) give rise to two homodimers (V_1: 2 HCA, V_3: 2 HCB) and one heterodimer (V_2: 1 HCA, 1 HCB), each with a distinct electrophoretic mobility and ATPAse activity. Both Ca^{+2} and actin activated myosin ATPase activity are higher for V_1 than V_3 (Pope et al, 1980) with the Ca^{+2} activated ATPase activity for $V_1V_2V_3$ in a ratio 6.4:3.7:1 (Hoh et al, 1977).

The ATPase activity is similar in both the left and right ventricle (Price et al, 1980), with the isoenzyme content typical of a given muscle. The isoenzyme content is not static but changes depending on the developmental status, hormonal treatment, and physioligical status of the animal (Clark et al, 1982; Hoh et al, 1977).

In addition to the light chains, each head of the myosin S-1 subunit contains two classes of sulphydryl groups, those that react quickly with sulphydryl directed agents (SH-1 groups) and those that react more slowly (SH-2 groups) (Reisler et al, 1974). These two thiols are ten residues apart in primary sequence corresponding to cysteine 10 and 20 of the P10 sequence (Elzinga and Collins, 1977), and appear to be relatively close to one another in the primary structure (12-14 A°) (Burke and Knight, 1980). Recent studies involving tryptic fragments of HMM indicate that these thiols are close to the junctional region between the rod and head portions of the molecule (Balint et al, 1978) and that the region containing the SH1 and SH2 is at or near the ATP binding site (Elzinga and Collins, 1977), the most probable distance being 38.7 A° (Tao and Lamkin, 1981). In addition the two groups are also in close proximity to the Ca^{+2} binding LC2 (Srivastava and Wilkman-Coffelt, 1980). Modification of either of these sulphydryl groups results in marked changes in the ATPase activities of the protein and the conformation of the myosin molecule. Reaction of the S1 group increases the Ca^{+2} ATPase activity and decreases the K^{+} EDTA activity. Once the S1 groups are bound, the binding of S2 groups inhibits Ca^{+2} ATPase activity (Scheuer and Bhan, 1979; Elzinga and Collins, 1977). The chemical modification of the SH1 and SH2 moieties of myosin heavy chains also result in the modification of light chain Ca^{+2} binding characteristics by decreasing the number of Ca^{+2} binding sites but not their affinity for Ca^{+2} (Srivastava and Wilkman-Coffelt, 1980). The conformational change in the S2 region is activated by ATP or several other nucleotides. F actin itself

has no effect on the conformational change but enhances the reactivity of S2 in the presence of MyATP yielding a cooperative interaction between F actin, MyATP and myosin (Kameyama, 1980). It should be noted that this enhancement is much smaller in cardiac than in skeletal myosin (Kameyama et al, 1980).

The Myosin Filament

Myosin is not found in a monomeric state in the myocardial cell, instead the protein is aggregated in a regular array known as the the thick filament. The myosin tails are wound together to form a rigid backbone from which the myosin heads project to make up the crossbridges (Katz, 1977). The crossbridges project from the surface every 14.3 nm along the filament with the surface lattice repeating every 429 nm (Squire, 1973), and are held in their proper orientation by the M line protein (Mannherz and Goody, 1976). Assuming the two elongated heads (crossbridges) are close to one another, Hasselgrove, (1980), has suggested that they twist the same way around the filaments but tilt in opposite directions, +30°, -30° so that they do not protrude from the filament but instead wrap around the surface. The first two crossbridges are arranged so that two at a given level project from the filament in opposite directions, and the next two crossbridges are rotated by 120° with this pattern repeating every three crossbridges (Mannherz and Goody, 1974). The crossbridges in resting muscle are perpendicular to the long axis of the thick filament, whereas in active muscle their tips shift toward the center of the sarcomere. This shift in orientation

of the crossbridge involves the flexible joint in the myosin molecule allowing the head to move toward the actin filament with the inner end of the head remaining close to the myosin backbone Hasselgrove, 1980).

The number of strands of the myosin filament is controversial. Huxley and Brown (1967), proposed a double stranded helix of cross bridges around each thick filament while data collected by Squire (1972), Tregar and Squire (1973), Hasselgrove (1980), and Morimoto and Harrington (1974), is consistent with a three or four stranded filament. Biochemical evidence indicates that the two stranded molecule where each subunit is represented by one myosin is incorrect because there is too much myosin to be accommodated in such a filament, (Hasselgrove and Rodger, 1980). Further research must be undertaken to determine whether the myosin filament is a three or four stranded filament.

II. Actin

Actin has been identified as the major protein of the thin filament (Katz, 1970). The actin filament exists in two forms, a low viscous monomeric globular protein, G actin, and F actin, a highly viscous fibrous polymer of G actin which interacts with the myosin molecule (Mannherz and Goody, 1974).

G-actin (MW 48,000) (Elzinga and Collins, 1973), is a globular protein containing approximately one half alpha helix (Katz, 1970). The polymerization of G actin to F actin can be effected by adding one of a number of salts to the solution. The action of salts to induce polymerization is non specific, that is a large number of

salts can cause polymerization (Katz, 1977). Gel filtration and centrifugation tecniques indicate the presence of a single high affinity site for divalent cations ($K= 10^5$ M-1). The high affinity site can bind Ca^{+2} and other divalent ions including Mn^{+2}, Sr^{+2}, Ni^{+2}, Mg^{+2}, and Zn^{+2}, which can readily replace one another on the G actin molecule (Strzelicka-Golaszewska, 1973). In the F form the exchangeability of the cation bound at this site is greatly reduced (Katz, 1977). In addition to the high affinity site, 5 low affinity sites with apparent association constants of 5-6 x 10^3 M^{-1} for Ca^{+2}, Mg^{+2}, Mn^{+2}, and Sr^{+2} have been found with five and eight sites for Ni^{+2} and Zn^{+2} (1.3×10^4 - 1.6×10^4 M^{-1}) respectively These low affinity sites are able to bind both monovalent and divalent cations.

There appears to be a correlation between the degree of actin polymerization and the number of moles of cation bound to the low affinity sites, suggesting that polymerization is a result of a decrease in the net negative charge and electrostatic repulsion of actin monomers upon binding of cations. It has been further suggested that the cation may maintain the monomers in a conformation appropriate for their specific interaction as a result of the nuetralization of negative charge (Strzelecka-Golaszewska et al, 1974).

The ATP molecule bound to monomeric actin also takes part in the polymerization of actin. G actin contains a single site for a nucleotide which preferentially binds ATP ($K= 10^{10}$ M^{-1})

(Engel et al, 1977), and will irreversibly denature in the absence of the bound nucleotide and cation. It has been widely suggested that the divalent cation participates in the binding of the ATP motiety since the affinity of actin for ATP increases when the high affinity Ca^{+2} binding site is occupied (Strzelecka-Golaszeweska, 1973). In the past the metal and nucleotide have been widely assumed to bind as a complex (Strzelecka-Golaszewska et al, 1974), but a recent NMR spectra study indicates that the binding sites on both G and F actin are seperated by at least 16 A° (Barden et al, 1980).

The polymerization of G actin-ATP is accompanied by dephosphorylation of the actin bound nucleotide so that F actin-ADP is formed. During polymerization as indicated above, exchangeable ATP is converted to nonexchangeable ADP, and P_i is liberated into the medium. Depolymerization is not accompanied by the reverse reaction, instead free ATP is substituted for the ADP previously bound to F actin giving rise to G actin-ATP in which the entire nucleotide is replaced (Katz, 1970). This irreversible dephosphorylation mechanism makes it possible that actin filaments can lengthen at one end and shorten simultaneously at the other as proposed by Wegner (1976). The irreversible nucleotide hydrolysis and filament polarity lead to a disparity in the critical concentration of the two filament ends. Thus, subunits are released from one end with the high critical concentration and incorporated in the end with low critical concentration. This process is referred to as head to tail polymerization (Wegner and Newhaus, 1981).

Differences in the rate of exchange of monomers and filament

subunits have been reported depending on whether the polymerization had been induced by $MgCl_2$, $CaCl_2$, or KCl, suggesting a possible role of these ions on polymer formation (Zechel, 1981). It is well established that actin is rapidly polymerized by millimolar concentrations of Mg^{+2} (Maruyami, 1981), while the rate of exchange of G actin is inversely proportional to the Ca^{+2} (Barden et al, 1980). This phenomenon has led to the suggestion that the exchange of ATP occurs through those molecules of G actin that are temporarily free of bound Ca^{+2} and that Mg^{+2} appears to be essential for polymerization (Katz, 1970). In addition, Zechel (1981), has determined that Mg^{+2} stabilizes the F-actin structure making it much more resistant to depolymerization than either Ca^{+2} or K^{+}.

Also affecting actin polymerization is a 97,000 D polymerization inhibiting factor that modulates both microtubule assembly and actin polymerization. It inhibits actin polymerization in the presence of relatively high but physiological concentrations of Mg^{+2} and Ca^{+2}, the lower the concentration of these ions the weaker the inhibitory effect (Nishida, 1981).

Actin exists in the sarcomere as the F actin polymer (MW 42000) in a globular ovoid shaped protein with an average diameter of 55 A°. The basic structure of both F actin and the thin filament is that of a double stranded helix. The distance between the nodes of F actin is 385 A° so that each half turn of the F actin filament contains approximately seven pairs of actin monomers (Katz, 1977). The tropomyosin molecules lie end to end in the grooves of the actin strands each spanning a length of seven actin monomers. Positioned

at intervals of 400 A° along the thin filaments are the units of the troponin complex, each unit consisting of one Ca^{+2} binding subunit, Tn-C, one inhibitory member, Tn-I, and one tropomyosin binding subunit, Tn-T. Together tropomyosin and the troponin complex have been termed the "regulatory proteins".

Tropomyosin

Tropomyosin (T) (MW 68,000) is an integral component of the contractile apparatus in skeletal and cardiac muscle tissue, where in conjunction with the troponin complex it functions as an element of the Ca^{+2} regulatory system for the control of actin myosin interaction (Ebashi et al, 1969). Consisting of two alpha helices in register in a parallel coiled coil (Cohen and Szent-Gyorgi, 1957; Sodek et al, 1972; McClaughlin and Stewart, 1975; Ohara et al, 1980; Stone and Smillie, 1978; Stewart, 1975; Johnson and Smillie, 1975), individual rod like tropomyosin molecules are linked head to tail with other tropomyosin molecules to form long polar filaments lying in each of the two grooves of the F actin structure (H.E. Huxley, 1972). Each tropomyosin molecule covers seven actin monomers on each of the two strands of F actin and also interacts with one complex of troponin (Tn-I, Tn-C, Tn-T) through the tropomyosin binding component Tn-T.

Data from electrophoretic studies show that two different subunits alpha (A) and beta (B) exist in skeletal muscle tropomyosin (Bodwell, 1967; Cummins and Perry, 1973; Johnson, 1974) which can be arranged as either AA or AB isomers (Eisenberg and Kielley, 1974). The ratio of B:A varies with the speed of contraction of the muscle,

the slow red muscles having a higher content of B subunits than the faster white ones. The data concerning cardiac muscle tropomyosin isomers is variable. Cummins and Perry (1974), found only A subunits in the heart which contradicts the skeletal muscle data since the myocardium is composed of slow red fibers. Leger et al, (1966) found that although B tropomyosin is present to the extent of 15-20% of the total tropomyosin in large slowly beating hearts (sheep, pigs, humans) it has been reported to be absent in the hearts of smaller animals (rabbit, guinea pig, rat). Previous reports have shown that the rabbit cardiac protein is similar to skeletal A tropomyosin in terms of its amino acid content, thiol content, immunological properties, peptide maps, and polyachromide electrophoretic gel mobilities (Leavis and Smillie, 1980).

Several systematic differences are apparent between the A and B subunits in amino acid content and sequence. The amino acid sequence of A and B polypeptide chains differ by 39 residues; 23 which are situated on the surface of the molecule (Wegner, 1980). Two differences are also apparent in the last nine residues near the C terminus where tropomyosin molecules form a polar end to end contact with the terminus of an adjacent tropomyosin molecule when bound along actin (Wegner, 1980). Finally the A chain has one cystiene residue present in a three fold excess over the B chains which contain two cystiene residues (Cummins and Perry, 1973). It should be noted that the difference in the affinity of the A and B tropomyosin for actin filaments as well as end to end contact of the two multiple forms of tropomyosin are small (Wegner, 1980).

Stereochemical observations indicate that the head to tail linkages connecting the tropomyosin molecules involves a short overlap of the broad faces of the supercoil for nine residues at the chain ends (COOH and NH_2) (McLauglin and Stewart 1975; Stewart and McLaughlin, 1975). The tropomyosin molecule composed of 284 amino acid residues (Stone et al, 1975) therefore has a structural repeat unit 275 amino acids long making 7-1/2 turns in one repeat length of 410 A° relative to actin (McLaughlin and Stewart, 1975). The two parallel A helices are held together by an interlocking zig zag arrangement of non polar amino acids on the helix surface. Hydrophobic amino acids at the core positions interlock with the corresponding positions on the other helix to form a close packed core while complimentary changes in the inner position on opposed helices form salt bridges that stabilize the structure (McLaughlin and Stewart, 1976)

Stewart and McLaughlin (1975, 1976) have observed that the distribution of negative charge along the tropomyosin sequence is highly periodic effectively dividing the molecules into alternate zones of strong negative and weak positive charge. Their results show the existence of fourteen bands consisting of 19-2/3 amino acids (11 negative, 9 positive) 1.29 A° apart with a 29.3 A° periodocity. The acid groups in the negative zones appear to play an important part in attaching tropomyosin to actin through numerous salt bridges which make direct links to basic groups on actin or by bridging indirectly to acidic groups via intervening Mg^{+2} or Ca^{+2} ions (McLaughlin and Stewart, 1975)

The two periods can be divided into two sets of seven A and 7 B

sites which alternate throughout the sequence. Each A or B site on the actin includes a surface region of 7-12 A° long with some non polar side chains which make contact with the tropomyosin positive zone (McLaughlin and Stewart, 1976). The A and B chains have about 284 residues and exhibit the expected regularities of the coiled coil structure which assigns each residue to a specific position of the repeating heptet (Edwards and Sykes, 1980). Although the general features of the A and B alternating bands are alike in general there appear to be systematic differences in detail between them suggesting that A and B bands represent different binding sites for actin monomers (Stone and Smillie, 1978) it has been proposed that seven boot shaped actin monomers on one strand of the helix lie within their broad heel ends in close contact with tropomyosin on that side of the groove (homostrand contact - A band) and the narrow toe ends of the actins of more distant strands reach across to make a more localized contact (heterostrand contact - B band) with the same tropomyosin at positions in between. This binding involves actins from both strands of the thin filament (binding constant 10^6-10^8) (Walsch and Wegner, 1980).

One important difference apparent between the two subunits is the more definite structural regularity of the A band compared to the B band. It has been proposed that the A band forms part of the binding site for the essential "off" position while the B band is used in the "on" position for the regulation of muscle control (McLaughlin and Stewart, 1976).

Troponin T

The Troponin-T (Tn-T) molecule is a globular and rodlike structure approximately 265 ± 40 A° in length (Flicker et al 1982), containing 259 amino acid residues (MW 30,503) (Pearlstone et al, 1976). Mild digestion with alpha chymotrypsin divides the Tn-T complex into two fragments T_1 (residues 1-158) and T_2 (residues 159-259) (Pato et al 1981, Ohtsuki, 1979), while further digestion in cyanobromide (CnBr) yeilds six CnBr fragments and the NH_2 terminal residues which like other myofibrillar proteins of rabbit skeletal muscle is acelylated (Pearlstone et al, 1976). In general, fifty percent of the amino acid residues are charged at a neutral pH, the COOH terminal sequence (221-259) is highly positively charged while the NH_2 terminal region (1-39) is highly acidic (Pearlstone et al, 1976). The secondary structure of Tn-T is 37% alpha helix and 10% beta sheet formation (Pearlstone and Smillie 1978, Pearlstone et al, 1976). Troponin-T is a very specialized molecule composed of distinct domains which carry out binding with three proteins tropomyosin, Tn-C and Tn-I.

Recent evidence indicates that a highly helical region (CB2 residues 71-151) of rabbit Tn-T is involved in the binding of the Tn-T complex close to or at the COOH terminal end (258-284) of the Alpha tropomyosin molecule (Nagano et al, 1980; Jackson et al, 1975). A second binding region of tropomyosin is present in the COOH terminal portion of Tn-T involving residues 197-259) (Pearlstone and Smillie, 1981). Since this region also binds to Tn-C it is likely that this part of the Tn-T molecule interacts in the vicinity of cysteine 190 or 1/3 of the distance from the COOH

terminal end of tropomyosin (Ueno and Ooi, 1977; 1978; Ueno, 1978). The binding of troponin to tropomyosin through the Tn-T component of the complex is independent of the presence of Mg^{+2} (Potter and Gergley, 1974) and Ca^{+2} (Kawasaki and Van Erd, 1972) but is affected by the ionic strength (Potter and Gergley, 1974; Kawasaki and Van Erd, 1972; Jackson et al, 1975).

The binding site of skeletal Tn-I to Tn-T spans two distinct regions of Tn-T the NH_2 terminal portion of Tn-T (residues 1-70) being very acidic at a neutral pH binds to Tn-I a basic molecule. The second region of Tn-I interaction encompasses residues 159-209 of Tn-T (Pearlstone and Smillie, 1980). Since the Tn-I binding site consists of two areas; residues 1-70 and 152-209, and because the tropomyosin binding site residues 71-151 are located between the two Tn-I sites it is possible that a folding of the Tn-T molecule brings the two Tn-I sites closer together for simultaneous interaction (Pearlstone and Smillie. 1980).

Tn-C also forms a complex with Tn-T (Perry and Cole, 1974). The Tn-C site spanning the COOH terminal region of Tn-T (residues 159-259) is either overlapping or adjacent to the second Tn-I site since fragment T_2 (159-209) binds to both Tn-C and Tn-I (Pearlstone and Smillie, 1978). This structure includes the highly basic COOH terminal segment of the sequence from 221-259, but also includes a region with more mixed charge properties extending from residue 159-220. These interactions with Tn-C are strengthened by the presence of Ca^{+2} ions and are unaffected by the addition of Mg^{+2} with this Tn-C binding region having very little alpha

helical or beta secondary structure. Immunoelectron microscopy (Ohtsuki, 1979), gel filtration, (Hitchcock et al, 1981) and elution volume studies (Horowitz et al, 1979) on the thin filament have shown that the Tn-T molecule may be somewhat elongated (10 nm). A subsequent study by Flicker et al (1982) has demonstrated that the Tn complex has both a globular and rod like domain, the tail portion measuring 160 $\pm$ 35 A° long and 20 A° wide. Furthermore, these studies have demonstrated a revised hypothetical three dimensional reconstruction of the Tn-T molecule and its constituent attachment sites. The T_1 region of Tn-T (1-158) contains two alpha helical segments, residues 90-148 and 60-81 which bind tightly to tropomyosin (Nagano and Miyamoto 1982, Nagano et al, 1980), confirming the studies of Jackson et al (1975), and Pearlstone and Smillie, (1980), which indicate that the tropomyosin molecule bound to Tn-T encompassing the region 71-151 was predicted to be 80% alpha helical in nature (Nagano et al, 1980). Pearlstone and Smillie (1977), indicate that the functional groups of Glu and Asp may play a role in the specific binding of Tn- T to Tm by maintaining the helicity of the Tn-T binding component which must be intact to bind to the tropomyosin molecule. One of the most feasible structures of the specific binding of Tn-T confirmed by computer model building techniques is that residues 1-59 form the globular portion at the end of the triple stranded stem of the troponin tropomyosin complex. Residues 149-259 also folded in a globular formation cover approximately half of the T_1 region and are connected to the Tn-T by C terminal residues 251-258 (Nagano and Ohtsuki, 1982). The T_2 region is the site for the interaction with Tn-C and Tn-I which

comprise most of the globular region of Tn which appears to be flattened to some extent.

Three phosphorylation sites have been identified on Tn-T; site I, site II, and site III (Moir et al, 1977), based on the observation that prolonged incubation with phosphorylase kinase yeilds a total of 3 mol of phosphate/mol of Tn-T (Perry and Cole, 1974). From the amino acid sequence determined by Pearlstone et al (1976), the phosphorylation of site I can be identified as serine I, site II as serine 149 or 150 and site III as serine 156 or 157 with the phosphorylation sites either very close to or possibly part of the protein protein interaction sites. Serine I is phosphorylated by the enzyme phosphorylase T kinase, while serine 149-150 and 156-157 are phosphorylated by phosphorylase kinase (Perry, 1979). Studies by Moir et al (1977) indicates that in Tn-T phosphorylation in vitro, only the N terminal site I is significantly phosphorylated, with a marked inhibition of Tn-T phosphorylation in the presence of Tn-C suggesting that interaction with Tn-C sterically blocks the phosphorylation sites, making them unavailable to the kinase (Moir et al, 1977; Pearlstone and Smillie, 1981). Although the function of Tn-T phosphorylation is still unclear, it has been suggested that it functions to alter the binding forces between components by changing the net charge at the interaction sites and thus modifying the function of the complex since as previously mentioned the phosphorylation sites are very close or part of the protein interaction sites (Pearlstone and Smillie, 1981).

Troponin-I

Cardiac (MW 23,550) and skeletal (MW 20,864) Tn-I are basic proteins that form a single polypeptide chain composed of 206 and 179 amino acid residues respectively (Burtnick et al 1975; Wilkinson and Grand, 1975). Cardiac muscle Tn-I contains an N terminal sequence of 26 additional amino acids not present in the skeletal muscle molecule which accounts for the larger size of the protein (Wilkinson and Grand, 1975). Circular dichromism and optic rotary dispersion methods conducted by Wu and Yang (1976) show that the secodary structure of Tn-I is 29% alpha helical and 20% beta sheet with charged amino acids accounting for 40% of the residues. Each protein possesses a net positive charge, eight for rabbit fast muscle and fourteen for rabbit cardiac Tn-I (Wilkinson and Grand, 1978).

By applying selective cleaving procedures a series of peptides can be isolated that span the whole molecule. Two regions of rabbit fast skeletal and cardiac muscle Tn-I have been implicated in interactions with other components of the thin filament (Syska et al, 1976). A study of the peptide fragments by electrophoresis in the presence of Tn-C and by affinity chromatography with Tn-C indicate that the N terminal regions (skeletal residues 1-47, cardiac residues 27-74), and the region consisting of residues 96-117 (cardiac 124-145) form complexes with Tn-C (Syska et al, 1976) through the involvement of particular side chains (Dalgarno et al, 1982). This suggests that Tn-C is able to interact with both regions of the Tn-I molecule represented by these peptides (Perry et al, 1979). The cross linking of Tn-C to Tn-I is Ca^{+2} sensitive,

in the absence of Ca^{+2} the extent of the TN-C - Tn-I cross linking decreases by 21-35% (Sutoh, 1980). When comparing this region in skeletal and cardiac muscle there is very little homology between the two, out of 48 residues only 19 are identical (Wilkinson and Grand, 1978).

In addition, residues 96-117 inhibit the Mg^{+2} stimulated ATPase of actomyosin a property that is potentiated by tropomyosin and is independent of Ca^{+2} concentration (Syska et al, 1976; Wilkinson and Grand, 1978). In the presence of Tn-C with which it forms a complex stabilized by Ca^{+2} (Head and Perry, 1974), the inhibitory activity of Tn-I is neutralized. It can be concluded that this region of Tn-I also interacts with actin and thereby prevents the interaction with myosin that is necessary for the high rate of ATP hydrolysis associated with contraction (Perry et al, 1979). It has been demonstrated that the inhibitory action of Tn-I is far more pronounced in skeletal (fast) muscle when compared to cardiac tissue (Perry, 1979). According to Talbot and Hodges (1981), this inhibitory region is one of the most strongly conserved sequences in any of the four regulatory proteins differing by five amino acids, four substitutions, and one insertion. The most striking substitution appears to be the replacement of arginine 113 by a leucine residue in cardiac Tn-I resulting in a dramatic decrease in inhibitory activity due to the loss of the charged side chain of arginine.

Tn-I from fast skeletal and cardiac muscle can be phosphorylated at specific sites by 3'5' cyclic AMP dependent protein kinase and phoshorylase kinase (Perry, 1979).

Phosphorylation of Tn-I from rabbit skeletal muscle by phosphorylase kinase occurs principally at threonine 11 (C-37) and phosphorylation by 3'5' cAMP dependant protein kinase is largely confined to serine 118 (C-146) (Moir et al, 1974; Huang et al 1974, Pearlstone et al, 1976). Phosphorylation of both of these positions is blocked in the presence of Tn-C, and it is doubtful if phosphorylation at these sites is of any physiological significance although they may play a part in the initial assembly of the Tn complex (Wilkinson and Grand, 1978; Weeks and Perry, 1977).

The portion of rabbit cardiac muscle Tn-I that contains the N terminal sequence of 26 amino acid residues not present in the skeletal muscle protein contains a serine residue at position 20 that is readily phosphorylated by 3'5' cAMP dependant protein kinase in vitro (Moir and Perry, 1977). When isolated from rabbit hearts by affinity chromatography more covalently bound phosphate (1.0-2.0 moles) is found when compared to skeletal Tn-I (0.4-0.5 moles) (Cole and Perry, 1975). From this it appears that the rapid phosphorylation of the serine 20 residue is responsible for the much higher rate of phosphorylation catalyzed by 3;5; cAMP dependant protein kinase in cardiac Tn-I compared to skeletal muscle protein. A study conducted by Moir et al, (1980) indicates that in normal perfused rabbit hearts serine 20 is 30-40% phosphorylated in the normal beating rabbit heart, and the phosphate on serine 20 is virtually the only phosphate that exchanges with the intracellular phosphate pool presumably in the form of ATP. In the normal perfused heart serine 20 appears to account for the total phosphate content of the N terminal peptide contributing 0.3-0.4 moles

phosphate/mole Tn-I.

The function of phosphorylation of Tn-I in cardiac muscle is twofold. It leads to a decrease in the sensitivity of the ATPase activity toward Ca^{+2} (Ray and England, 1976, Reddy, 1976; Perry et al, 1979; Balin, 1979), and it may smooth out the contractile response of the heart produced by the transient increase in Ca^{+2} concentration resulting from the adminstration of adrenalin or other B adrenergic agents (Perry, 1979).

Tropnin-C

Skeletal muscle is a single acidic chain of amino residues (MW 18,000) composed of four homologous regions numbered I-IV from the N terminus (Collins and Elzinga, 1975; Collins, 1976). Each region consists of a ten residue Ca^{+2} binding loop rich in glutamine and aspartine, that contain carboxylate groups which act as key metal coordinating ligands (McCubbin and Kay, 1980). They are flanked on either side alpha helical regions, with the whole structure stabilized by interactions between specific hydrophobic side chains of the helix (Wilkinson, 1980).

Potter and Gergley (1975) and Potter et al (1977) reported that rabbit skeletal Tn-C binds Ca^{+2} and Mg^{+2}. Tn-C binds 4 mol Ca^{+2}, with two high affinity sites that bind Ca^{+2} and Mg^{+2} competitively (Ca^{+2} Mg^{+2} sites) and two with lower affinity which bind Ca^{+2} exclusively (Ca^{+2} specific sites). Further studies by Leavis et al (1978) utilizing circular dichromism (CD) and fluorescent titrations have determined that sites I and II

are the low affinity Ca^{+2} specific sites while sites III and IV are the high affinity sites of Tn-C.

The trigger for the series of structural events that take place during regulation concerns the binding of Ca^{+2} by the Tn-C complex. This metal binding is accompanied by a conformational change in the protein molecule which is transferred through the quaternary structure of the entire multiprotein relaxing system (McCubbin and Kay, 1980). Data presented by Johnson et al, (1979) and McCubbin and Kay (1980) indicate that about 70% of the conformational change is elicited by the high affinity Ca^{+2} binding sites and some 25% of the conformational change is produced by the low affinity binding sites.

The binding of Ca^{+2} to the low affinity sites (I and II) results in a subtle alteration of the tertiary fold of the N terminal half of Tn-C involving weakened contacts between several hydrophobic groups by increasing the interatomic distance between interacting phenylalanine and methyl groups (Evans et al, 1980). The binding of Ca^{+2} to the $Ca^{+2}Mg^{+2}$ sites produces a large enhancement of intrinsic tyrosine fluoresence and circular dichroism in the 200-400 nm region (Murray and Kay, 1972;), the latter indicating an increase in the alpha helix content from approximately 30% of the residues in the divalent cation protein to 50% with the saturation of the $Ca^{+2}Mg^{+2}$ sites. Further hydrodynamic measurements indicate that a compacting of the molecule and increase in the ordered structure also occurs upon binding of Ca^{+2} to the high affinity regions of Tn- C. Finally McCubbin and Kay (1980) using ultraviolet difference absorption spectroscopy

demonstrated that the environment of one or more tyrosine residues have become more nonpolar consistent with the compacting of the molecule which also appears to be accompanied by neutralization of negatively charged carboxyl groups..

As previously mentioned, most of the structural changes take place on the binding of divalent cations ($Ca^{+2}Mg^{+2}$) to the high affinity sites. However binding to the low affinity sites is accompanied by additional minor changes in helical content, tyrosine fluorescence and the environment of hydrophobic residues (Johnson et al, 1979; Levine et al, 1978). From this data it has been suggested that Ca^{+2} or Mg^{+2} binding to the high affinity sites presumably stabilizes the structure of the protein and determines its secondary and tertiary structure (Perry et al, 1972; Potter and Gergley, 1975). It may maintain it in a conformation which is ready for the regulatory event of Ca^{+2} on the Ca^{+2} specific sites (McCubbin and Kay, 1980) which are often termed the regulatory sites (Potter and Gergley, 1975).

The protein chain of cardiac Tn-C is composed of 161 amino acid residues (MW 18,450) (Van Erd and Takahashi, 1976). There are fifty five replacements and two additional amino acids when compared to rabbit skeletal Tn-C. The protein is strongly acidic with an excess of thirty negatively charged groups. Van Erd and Takahashi (1975) determined that the amino acid sequence of three of the corresponing sites (II, III, and IV) were very similar to the corresponding sequences in rabbit skeletal Tn-C. The fourth region (I) (res 28-40) contained seven amino acid replacements and one additonal amino acid residue involving the key metal coordinating Asp and Glu

residues, therefore concluding that this site had probably lost its ability to bind Ca^{+2}. Further Ca^{+2} binding studies and Ca^{+2} induced changes in the alpha helix of tyrosine fluorescence in cardiac muscle have confirmed the presence of three sites, two high affinity $Ca^{+2}Mg^{+2}$ sites essentially identical to sites III and IV of skeletal Tn-C (Leavis and Kraft, 1978; Potter, 1977, Potter et al, 1977). Equilibrium dialysis (Potter, 1977; Potter et al, 1977) and ion selective electrode studies (Leavis and Kraft, 1978) suggest that cardiac Tn-C has one low affinity Ca^{+2} specific site corresponding to site II. It has been determined that this Ca^{+2} specific site is similar in terms of Ca^{+2} affinity, specificity, and Ca^{+2} coordinating amino acids (Van Erd and Takahashi, 1975; Potter et al, 1977) to the two Ca^{+2} specific regulatory sites of skeletal Tn- C.

Studies conducted by Johnson et al, (1980) indicate that cardiac Tn-C binds Ca^{+2}, undergoes Ca^{+2} induced increases in alpha helix, and forms a complex with other Tn subunits as does skeletal Tn-C. The Ca^{+2} specific site on cardiac Tn-C appears to be responsible for the regulation of cardiac muscle ATPase activity and contraction while the two high affinity sites maintain the stability of the complex as it does in skeletal Tn-C (Potter, 1977; Leavis and Kraft, 1978; Leavis et al, 1978; Burtnick and Kay, 1977).

As well as inducing conformational changes in the Tn complex Tn C also functions to 1) neutralize the inhibition of Mg^{+2}-stimulated ATPase of actomyosin inhibited by Tn-I 2) inhibit

phosphorylation of fast skeletal muscle Tn-I by 3'5' cAMP dependent kinase and phosphorylase kinase in skeletal muscle (but not in cardiac) 3) to form a stable complex with Tn-I in the presence of Ca^{+2} 4) to form a Ca^{+2} insensitive complex to Tn-T.

Gel Electrophoresis studies have identified one site of Tn-C that is able to form a complex with Tn-T (Jackson et al, 1975) although the specific site is still controversial. Leavis et al (1978) concluded that Tn-T binds to region IV (residues 121-159) while Grabarek et al (1981) have determined that Tn-T binds to the NH_2 terminal half of Tn-C. Both studies confirm that Tn-C-Tn-T binding is not Ca^{+2} dependent, thus the Tn molecule is always anchored to the thin filament.

Tn-C is also able to form a stable complex with Tn-I, an interaction that requires Ca^{+2} for its formation (Head and Perry 1974). Further studies with proteolytic fragments of Tn-C have, suggested at least two sites of interaction with Tn-I (Syska et al, 1976), a view supported by recent work on the reactivity of lysine residues by Tn-C (Hitchcock et al, 1981). Leavis et al (1978) have shown both by formation gels and by fluorescence titration that site III interacts strongly with Tn-I (res 89-120) while site II (46-77) shows a weaker interaction, coinciding with the results of Weeks and Perry (1978) and Perry et al (1975). Both regions II and III require the presence of Ca^{+2} to form complexes with Tn-I suggesting that a specific Ca^{+2} dependent spatial arrangement of residues is involved in the binding essential for the interaction to occur (Grabarek et al, 1981). More specifically, Perry et al (1979) suggests that the conformational changes that occur on Ca^{+2}

binding bring the adjacent negatively charged residues not involved in cation binding into a more favorable position for interaction of the active peptide isolated from Tn-C with Tn-I indicating that aspartic and glutamic acid residues involved in the binding of Ca^{+2} may not be directly implicated in the interaction between the two troponin components (Perry, 1979).

The Tn-C-Tn-I sites of interaction are proposed to share several features. First, all binding occurs on the alpha helix on the NH_2 terminal side of the binding domains (Grabarek et al, 1981). Second, all contain a remarkably similar cluster of acidic residues along one surface of the helix. Since earlier work on Tn-I fragments identified two peptides from Tn-I that form complexes with Tn-C and have a high concentration of basic amino acid residues it has been speculated that the bonds between the two are electrostatic (McCubbin and Kay, 1980). Third the lysines in the binding regions all exhibit reduced reactivities in the complex with Tn-I (Hitchcock et al, 1981; Grabarek et al, 1981).

Residues 83-134 which are involved in forming one of the Ca^{+2} dependent complexes with Tn-I function to neutralize the inhibition of Tn-I on the Mg^{+2} stimulated ATPase of desensitized actomyosin. The peptide also inhibits the phosphorylation of fast skeletal muscle but not cardiac muscle Tn I by 3'5' cAMP dependent protein kinase (Weeks and Perry, 1978).

Two possible mechanisms have been suggested for the manner in which Tn-C neutralizes the activity of Tn-I (Perry et al, 1975). In the first scheme Tn-C interacts with Tn-I in the N terminal region in a way that does not physically block the region of the inhibitory

peptide. By a conformational change induced as a result of the interaction the conformation of the inhibitory peptide region becomes modified so that it can no longer interact with actin nor can the adjacent residue at postion 118 be phosphorylated by 3'5' cAMP dependent protein kinase. In the alternate scheme the folding of the polypeptide chain in Tn-I is such that the N terminal region and that of the inhibitory peptide are adjacent to the surface of the protein. Thus, the interaction of Tn-I with Tn-C would effectively render the inhibitory peptide region unavailable for phosphorylation or interaction with actin (Perry, 1973).

FUNCTION

Introduction

In resting muscle the free Ca^{+2} concentration is lower than 10^{-7} M and the concerted action of the tropomyosin-troponin system results in an inhibition of the Mg^{+2} stimulated ATPase activity of the myofibril (Adams and Schwartz, 1980). Cardiac contraction is initiated when an action potential occurs across the sarcolemma. The small amount of Ca^{+2} that crosses the sarcolemma during the action potential triggers a Ca^{+2} release mechanism from the terminal cisternae of the sarcoplasmic reticulum, raising the Ca^{+2} concentration in the sarcoplasm to 10^{-5} (Fabiato and Fabiato, 1977). The Ca^{+2} diffuses to the troponin molecules of the thin filaments, and at this Ca^{+2} concentration (10^{-5} M) binds to the Tn-C complex altering the conformation of Tn-C. This removes the inhibition of Tn-I and tropomyosin on Mg^{+2} stimulated ATPase activity by permitting the exposure of the myosin binding site on actin (Squire, 1974).

Muscle contraction consists of the cyclic attachment and detachment of the globular portion of the myosin molecule to the actin filament which results in the sliding of the filaments past one another. The energy for this process is provided by the hydrolysis of ATP following activation of the myosin ATPase enzyme. Relaxation is initiated when the SR accumulates Ca^{+2} ions permitting disociation of the Ca^{+2} ions from the troponin complex. The contraction-relaxation cycle is the topic of the following section.

Physiological Mechanisms of Muscular Contraction

In 1954 H.E. Huxley and Hanson and A.F. Huxley and Niedergerke independently developed the sliding filament theory which has provided a basis for the physical and chemical events of muscular contraction. The theory suggests that contraction is brought about when the interdigitating thick and thin filaments slide past one another without a change in length in either filament. Shortening of the muscle fiber and tension development occurs as the result of cyclic reaction between the projections of the myosin filaments and active sites on the actin filaments. The myosin projection attaches to the thin filament to form a crossbridge and presumably swivels to a different angle pulling the thin filaments past the thick. The crossbridges on opposite sides of the M line swivel in opposite directions which pull the filaments into a greater overlap towards the center of the sarcomere, thus decreasing the distance between Z lines and shortening the muscle. In order for significant shortening each crossbridge must act in a cycle; it must attach, swivel, detach, and then reattach at a point farther along the thin filament with a single cycle causing a relative movement of the two filaments by about 100 A°.

In 1957, H.E. Huxley added to this theory by incorporating the force velocity curve and the relation between load and the rate of energy liberation by A.V. Hill (1938) into his original sliding filament theory.

Relation to A.V. Hill's Theory

In 1938 A.V. Hill determined 1) that the rate of liberation of heat increases linearly with the speed of contraction 2) the increased

rate of energy liberation flattens off at higher speeds of shortening indicating that the energy liberated per change of length decreases as the speed of shortening increases 3) a fall in energy liberation occurs when a muscle is stretched during contraction.

To explain these phenomonen, Huxley proposed that the individual crossbridges undergo cycles in which they attach, generate force, and detach, with detachment presumably brought about by interaction with ATP. Relating to the increased energy release when shortening is permitted, he suggested that detachment (g) is slow unless shortening occurs and the sliding movement allows the crpssbridge to complete the working part of its stroke. The rate constant for attachment (f) of a free crossbridge is moderate in relation to the speed of shortening to explain the fact that as the speed of shortening increases the energy liberated per change in length becomes smaller. Thus at low speeds of shortening each crossbridge has sufficient time to attach to each site of actin that is within range as the filaments slide past one another. At higher speeds of shortening the rate constant for attachment becomes rate limiting, and sufficient time is not available for the crossbridge to attach to each site of actin. In explaining the reduced energy liberation during stretch H.E. Huxley (1957) made the suggestion that the attachment process was reversible without the splitting of ATP if the crossbridge was prevented from going through its working stroke.

In his 1957 model H.E. Huxley proposes that actin and myosin are initially detached with myosin oscillating back and forth about its equilibrium position as a result of thermal agitation

(Brownian motion). If actin appears to be within the range where the combination of myosin with actin is catalyzed attachment may take place. When this happens the tension on the elastic element is exerted on the actin thread. The extent of movement between the two filaments is limited by an elastic connection which Huxley assumes is located in the crossbridge. A second variable introduced in the model is denoted "x", a measure of the position of the actin site relative to the crossbridge which decreases at a constant rate during steady state shortening.

Relation to the force-velocity curve

One further addition to the Huxley (1957) model is the notion that a crossbridge can exert either a positive or negative force. Huxley proposed that many steady state properties of muscle could be explained by having the crossbridges attach at a moderate rate (f) and detach slowly (g) in a region where they exert positive force and detach rapidly where they exert negative force. It has been suggested that most of the force-velocity curve is determined by the value f. Only when the velocity is nearly maximum is the slope affected by the detachment rate.

The force-velocity curve indicates that as the velocity of steady state isotonic contraction increases, the force (P) exerted by a muscle decreases in a hyperbolic fashion. At both zero velocity (maximum force) and maximum velocity (zero force) the muscle does no work, but at intermediate velocities the rate of work production increases to maximum at about 1/3 Vmax decreasing again at higher velocities.

Where the attachment of the crossbridge is postulated to be

relatively slow there is a marked decrease in the number of attached crossbridges that exert positive force as the velocity increases, therefore in Huxley's model the decrease in force with increased velocity is primarily due to the slow attachment rate. Consequently the model predicts (as does Huxley and Simmons, 1971) that the number of attached crossbridges will decrease as velocity increases.

H.E. Huxley (1969) added to this model by presenting a simple structural model of the cycle which has become the basis for later physiological models. The essence of this model is a preferential attachment of the crossbridge at some angle θ_1, rotation through the angle from θ_1-θ_2, and detachment at angle θ_2. The proposed angles are 90° and 135° with relation to the axis of the thin filament pointing toward the end of the sarcomere. In addition Huxley assumed that the myosin head attaches to the myosin filament by two flexible joints, one at the junction of S-2 with LMM and the second at the junction of the S-1 S-2 parts of the molecule, a fact later confirmed by Mendelson et al, (1973). This allows the orientation of the head of the molecule to be maintained; the flexibility of the joint allows the link to swing further out from the backbone of the filament by bending at the HMM-LMM junction allowing direct myosin-actin interaction to take place over a wide range of interfilament spacing. The occurrence of the two joints gives the unattached crossbridges considerable azithmuthal and radial flexibility which Huxley points out is a necessary property of the crossbridge. As the muscle shortens over a relatively long distance the distance between the filaments changes markedly while the force exerted per crossbridge remains constant.

The Podolsky-Nolan Model

Rather than adjusting the crossbridge parameters to fit the force-velocity relation, Podolsky and Nolan (1971) attempted to fit the nonsteady motions that precede steady shortening after step changes in load, discovered after Huxley's 1957 model (Podolsky, 1960; Civan and Podolsky, 1966).

Relation to step changes in load

The idea of investigating contraction processes by imposing a sudden change of mechanical conditions during a contraction is not new. Gasser and Hill (1924) and Levin and Wymann (1927) utilized the transient responses recorded when the load on the muscle fiber or length was suddenly changed to determine that active muscle can be represented as two components in series, a contractile component and a series elastic component. The contractile component was defined as having a definite force-velocity curve that at any instant its speed of shortening was determined initially by the load of the muscle at that instant. Similarly, the series elastic component was defined as having a length that was entirely determined by the load at that instant.

Podolsky (1960) and Civan and Podolsky (1966) were the first to show that the responses of muscle to sudden changes of load or length were much more complicated than those of the two component model. They determined that there was an initial shortening as would be expected from a series of changes (velocity transient) with no consistant pattern as predicted by the two component model. At first the speed of shortening was several times higher than the steady state value for the load, declining to a lower value, to zero

or reversing its direction, finally building up slowly to its steady state value.

To explain this data Podolsky and Nolan (1971) proposed a model with only one attached state in which the rapid redevelopment of force following a quick release is due to the attachment of new crossbridges. A small shortening step allows some of the crossbridges to transfer from a situation where they exert little or no force to one in which they exert substantial force. This transition is made by the attachment of crossbridges free in the isometric state. The model postulates 1) a rapid attachment of the crossbridges in regions of positive force and relatively slow detachment following net ATP hydrolysis in regions of negative force 2) an increased number of crossbridges and therefore an increased stiffness at the end of rapid early recovery.

Huxley and Simmons Model

Huxley and Simmons (1971) utilized the converse experiment of Podolsky and Nolan (1971) where a sudden small change is imposed during an isometric contraction and the ensuing tension changes are recorded. They determined that tension undergoes a relatively large alteration simultaneously with the step changes in length, recovering quickly towards a level closer to that which existed before the step. The final recovery to the original tension takes place on a much slower time scale. In essence they found a tension change simultaneous with the length change which varied in direct proportion to the amount of overlap and therefore to the number of crossbridges capable of contributing to tension. On these grounds Huxley and Simmons (1971) proposed that the majority of the

instantaneous elasticity resides in the crossbridge, and that this instantaneous elasticity is attributed to the simultaneous drop in tension and simultaneous shortening found in their L-T curves. Further to this they proposed that in addition to the instantaneous elastic element the crossbridge contains an element with viscous and elastic properties that can maintain tension while taking up limited but substantial amounts of length change accounting for the early tension recovery. The gradual recovery of tension was accounted for by detachment and reattachment of the crossbridge with kinetics, similar to those postulated in the 1957 model. By placing the elastic element in the crossbridge they have made it possible for each of the crossbridge states to exist over a wide range of axial positions (x) first introduced by A.F. Huxley (1957). The attached crossbridge can then act as a spring. From their quick release studies Huxley and Simmons (1971) determined that it can be stretched or compressed more than 40 A°, therefore their revised model proposed that when the filaments move past one another the spring S-2 exerts either positive or negative force. The angle of the crossbridge head does not change as the crossbridge moves along x, rather a change in state causes a change in angle of the attached crossbridge. When transitions between the two attached states occur the independent elastic element in the crossbridge is either stretched of compressed. Since the 1957 theory does not account for the transient responses that are seen when the length load is suddenly altered during a contractrion, Huxley and Simmons (1971) favor the production of tension occuring as a step distinct form and subsequent to the attachment itself.

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A Comparison of the Two Models

Several similarities exist between the Podolsky and Nolan model and the Huxley and Simmons models. The similarities include 1) a small shortening step allows some of the crossbridges to transfer from a situation where they exert little force to one in which they exert a substantial force 2) the rate constant for this transfer increases with the size of the shortening step 3) rapid detachment does not begin rapidly after the start of shortening. The differences between the models include 1) In the Podolsky-Nolan model the transition during the shortening step is made by the attachment of crossbridges which were free in the isometric state while in the Huxley-Simmons model the crossbridges are attached throughout and transfer from one attached state to another to exert force. Unlike the Huxley-Simmons theory the Podolsky-Nolan theory predicts an increased number of attached crossbridges and therefore increased stiffness at the end of the early rapid recovery 2) Podolsky and Nolan also show the number of attached crossbridges should be greater during steady shortening than in the isometric condition (Huxley and Simmons, 1971).

The Eisenberg and Hill Model

Eisenberg and Hill (1978), modified the Huxley-Simmons model so that the crossbridge is not only elastic but by changing state can exert force. The force can be developed when one state transforms to another, but positive work is done only when the crossbridge in a single attached state moves along x as the free energy drops. Introduced into their model is "angular free energy" defined as the free energy in the interaction of the myosin head with actin and

"structural free energy" defined as the free energy stored in the independent elastic element localized in S-2. Unlike the Huxley-Simmons model where the angular free energy wells are narrow Eisenberg and Hill (1978) suggest that the angular free energy wells might be much broader. In this way a change in angular free energy rather than one dimensional Brownian motion could stretch the elastic element during a change in state. The independent elastic element can then be discarded while the crossbridge elasticity is provided by the broad angular free energy wells. In such a model the force is exerted directly by interaction between the crossbridge head and actin rather than indirectly via the independent elastic element. As the filaments slide past each other the elastic element is not stretched, the attached crossbridge simply changes angle exerting either positive or negative force. Unlike the Huxley-Simmons model where the angle of the crossbridge head does not change as the crossbridge moves along x, in the Eisenberg and Hill model the cross bridge head smoothly rotates through varying angles as its axial position or x value changes. Because there is no independent elastic element in this model there is no structural free energy, rather the total free energy of the crossbridge is equal to the angular free energy which by itself provides elasticity of the crossbridge. As the muscle shortens or is stretched movement of the crossbridge states provides the elasticity which in the Huxley-Simmons model was provided by the independent elastic element.

To summarize, the key difference between the Huxley-Simmons model and the Eisenberg-Hill model is that a change in state causes

a change in the angle of the attached crossbridge while in the Eisenberg and Hill model transition between the two states occurs without any change in angle.

Cardiac Muscle Mechanics

On the basis of mechanical properties described for skeletal muscle several conceptual models of contraction mechanics have been developed for the isolated cardiac muscle (Sonnenblick, 1962; Minelli et al, 1975). Most studies utilize a three component model consisting of an active contractile element (CE) which is arranged in series with a passive elastic element (SE), both of these components being coupled in parallel with another elastic element (PE). This component does not appear to participate directly in contraction but is considered to contribute to the resting tension of the muscle as a non linear function of its length (Sonnenblick, 1962).

On stimulation after a short latency the CE is fully activated rapidly attaining its maximum capacity to resist stretching, as well as its maximum ability to develop force and shorten. During an isometric contraction it shortens and stretches the SE although the overall muscle length remains constant, thus delivering force to the muscles external fixations. The SE element functions to dampen the force generated by CE. Therefore peak tension does not equal the CE, instead peak tension occurs when the tension exerted by SE is just equal to the tension under which CE will neither lengthen nor shorten during activation. Under these circumstances the peak tension represents the active state (AS). The active state is defined as a measure of the mechanical energy derived from

chemical reactions in the contractile element whether measured in terms of a capacity to develop force or an ability to shorten (Adams and Schwartz, 1980). Changes in the rate of tension development (dp/dt) and the magnitude of developed tension are directly related with the intensity of AS.

The delay of force development observed in cardiac and skeletal muscle is due to the time necessary for the contractile element to shorten and stretch the SE element. Force builds up in the SE element as it becomes stretched according to stretch strain characteristics. The velocity of shortenting falls as the force (P) against which it is shortening is increased. This property of the CE is denoted by the force-velocity relations. The rate of force deveopment dp/dt is then uniquely determined by the interaction of the activated contractile element and the SE component.

As in other types of muscle the actively developed tension of heart muscle is a function of its length prior to the onset of contraction. This phenomonen in the intact heart forms the basis of the Frank Starling principle (Sonnenblick, 1962). The length tension relationship in skeletal muscle contends that with an increase in length of the muscle from an initial unstretched state the degree of overlap and consequently the number of potentially active actomyosin complexes increases with a greater number of actomyosin complexes formed as the tension increases. Further increases in length result in a reduction in the amount of active tension production as the optimal degree of filament overlap is surpassed. At any given fiber length and degree of myofilament

overlap, alterations in the state of activity and changes in tension development depend on the number and intensity of actomyosin complexes as well as an increase in the rate and duration of complex formation.

The relatively straight forward nature of the time and length dependent features of skeletal muscle are in marked contrast to the situation in the heart. As already mentioned sudden stretching of skeletal muscle reveals a rapid onset of the active state which manifests as a plateau of tension early in the course of the twitch. In cardiac muscle the active state is slow in onset and thus is time dependent with no plateau observed after quick stretch - the contractile event lasts several hundered milliseconds as opposed to the much shorter twitch of a skeletal muscle. The inability of the length tension to account for the activation process in cardiac muscle occurs at the short sarcomere lengths (Katz, 1977) where the length tension relationship is poorly understood.

Relationship Between Velocity of shortening and ATPase Activity

Before leaving the physiological aspects of muscle contraction one further relationship must be eludicated, namely the Vmax-ATPase relation in ventricular muscle. It is well documented that the velocity of unloaded shortening (Vmax) has a positive correlation with myosin ATPase in both skeletal (Barany, 1967) and cardiac muscle (Carey et al, 1979; Hamrell and Low, 1978). The implications of such a correlation is that the rate of unloaded muscle shortening is related to the ability of the myosin molecule to release chemical energy by hydrolyzing ATP. This phenomonen is in agreement with the experiments of A.V. Hill (1938) on the mechanics and heat production

of skeletal muscle. A.V. Hill demonstrated that the rate of heat production was a function of the velocity of contraction and implied that the rate of energy release was related to the velocity of shortening.

The Biochemistry of Muscle Contraction

The Eisenberg and Moos Model

In studies on the steady state ATPase activity of acto HMM and acto S-1 it has been found that a linear relationship exists when the reciprocal of the ATPase rate is plotted against the reciprocal of the added actin concentration (Eisenberg and Moos, 1968; 1970). The intercepts of this double reciprocal plot yeild values for the maximal actin activated ATPase rate (Vmax) and the actin concentration required for 1/2 maximal ATPase activity (Kapp). From the intercepts of these plots it was determined with Mg^{+2} present at low ionic strength that the acto HMM is about one hundred times more dissociated in the presence of ATP. Further studies by Eisenberg and co-workers (1968) determined that acto S-1 is capable of hydrolyzing ATP and being dissociated by ATP. Since S-1 was postulated to have a single binding site for ATP they concluded that the same ATP site is involved in both of these processes.

To account for these results Eisenberg and Moos (1968) and Eisenberg et al (1968) proposed the kinetic model shown in Figure IV where M=myosin, A=actin, T=ATP and D=ADP. In this model, the first reaction to occur is the combination of myosin ATP with actin to form the actin-myosin-ATP complex. In the second reaction or

motion producing action, actin activates the splitting of bound ATP, and the ternary A-M-T complex is formed with the bridge in the 90° configuration so that motion will occur when it transforms to the rigor state. The driving force for this motion producing reaction comes from both the conversion of bound ATP into bound ADP and from the transformation of the relatively weak bond between actin and myosin ATP to the much stronger rigor actin myosin ADP bond. In the third reaction the dissociation of the rigor actin myosin bond is coupled energetically with the replacement of ADP by ATP on the hydrolytic site of the myosin crossbridge.

Although this model explains the steady state ATPase activity of acto S-1 and acto HMM, it does not take into account the pre-steady state behavior of myosin alone. Several studies indicate that once ATP binds to myosin it is hydrolyzed to form bound ATP and P_i on the surface of the enzyme in the presence of Ca^{+2} and Mg^{+2}. This ATP hydrolysis occurs much more rapidly than the ADP and P_i are subsequently released, and has been termed the initial P_i burst (Lymn and Taylor, 1970; Taylor et al, 1970; Tonumura et al, 1969), which had not been accounted for in the Eisenberg and Moos model.

In addition, the Eisenberg and Moos model did not provide a mechanism for the binding of ATP to detach the myosin from actin during each cycle of ATP hydrolysis. The first evidence that dissociation and reassociation of the actin and myosin filaments occured each time ATP was hydrolyzed came from the work of Lymn and Taylor (1971). Their pre-steady state experiments show that when ATP was added to a complex of actin and HMM, dissociation of the actin

HMM complex by ATP occurred before the initial burst of ATP hydrolysis (P_i burst) on the HMM head. These workers also determined that at low actin concentrations the rate of dissociation of ATP was much faster than the rate of ATP hydrolysis in the initial P_i burst. Lymn and Taylor (1971) thus proposed a model (Figure I (2) to account for both the P_i burst and the association and reassociation of the actin and myosin filaments not included in the Eisenberg and Moos model.

To summarize, the major features of this model are 1) the dissociation of the acto HMM complex by ATP (A-M-T>M-T) precedes hydrolysis of the ATP on the HMM in the initial P_i burst (M-T>M-D-P) ie: the hydrolysis step occurs after HMM is dissociated from actin 2) it requires S-1 to detach from actin each time an ATP molecule is hydrolyzed because the initial P_i burst is postulated to occur only when the S-1 fragment is detached from actin 3) the major rate limiting step in the cycle is a relatively slow release of products occuring after the HMM rebinds actin. Thus this model as originally presented suggests that at high actin concentrations a large fragment of the HMM or S-1 would remain complexed with actin even in the presence of ATP.

One of the most attractive features of this model is that it provides a possible mechanism for the cyclic dissociation and reassociation of the myosin crossbridge with actin. Before ATP binds the cross bridge is strongly attached to actin at a 45° angle. When ATP binds the binding of the crossbridge to actin is weakened and detaches. Only when the initial P_i burst occurs is the cross bridge able to reattach to actin presumably at a 90° angle. Finally

as ADP and P_i are released the attached crossbridge rotates from a 90° angle back to a 45° angle simultaneously doing work (Adelstein and Eisenberg, 1980).

As mentioned, one of the major predictions of the Lymn-Taylor model was that the rate limiting step in the ATPase cycle was the release of products which occurred after the myosin product complex rebound to actin (A-M-P-P_i > A-M). This led to the prediction that almost all of the HMM or S-1 should be complexed with actin when the ATPase is close to Vmax. Contrary to this prediction viscosity, turbidity, quasi electric light scattering, ultracentrifuge, and kinetic studies have demonstrated that even under conditions where the actin activated ATPase is close to its maximal value (Vmax) a large fraction of the HMM or S-1 remains dissociated from the actin (Eisenberg et al, 1972b; Fraser et al, 1975; Mulhern and Eisenberg; 1976 Eisenberg and Kielly, 1972). On this basis they proposed that in the presence of ATP the myosin head can exist in two conformations. One of these conformations they called the refractory state since it seemed to be unable to bind to actin at any concentration of actin obtainable in vitro. The other conformation they called the non refractory state since it was able to bind actin thus accounting for the actin activation of the HMM. Their model suggests that both of the HMM heads act independently. Presumably on a random basis as one head of HMM goes through the cycle the other head will remain refractory so that only one head of the HMM binds to actin at a time.

The refractory state model is identical with the Lymn-Taylor model except that an additional step, the transition from the

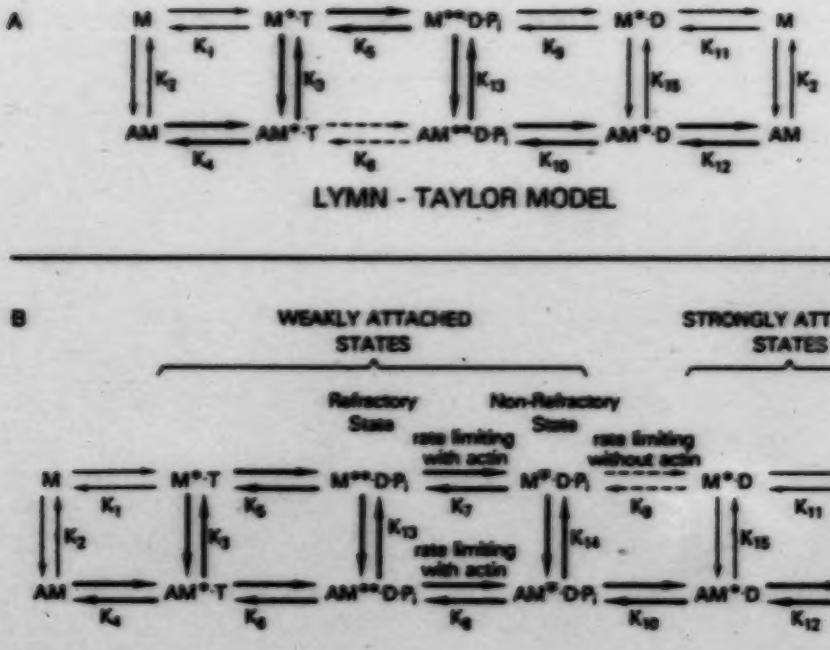

Figure IV Refractory State Model (Stein et al, 1979)

refractory to the non refractory state was added following the initial P_i burst (Figure I (3)). Like the initial P_i burst this step could only occur when S-1 was detached from actin. In contrast to the Lymn-Taylor model the myosin remains in the dissociated or refractory state for most of the cycle until a rate limiting transition to the non refractory state occurs. Only then can rebinding to actin occur which in turn causes the rapid release of products. Thus in contrast to the slow product release suggested by the Lymn-Taylor model the major rate limiting step in the refractory state model is the transition from the refractory to the non refractory state that occurs before the myosin head rebinds to actin.

The refratory state model provides a biochemical explanation for H.E. Huxley's (1957) model of crossbridge action where a slow rate of detachment of the crossbridge to F actin was quite slow, important in determining a large part of the force velocity curve and explaining the levelling off of ATP turnover rate at high velocity. The slow transition from the refractory to the non refractory state may explain the slow detachment of the crossbridge in vivo. In the refractory state model the rate of product release which occurs after S-1 reattaches to actin is rapid, consistent with the brief time a crossbridge spends attached in a rapidly shortening muscle.

Modified Refractory State Model

The modified refractory state model in its simplest form predicts that at saturating actin concentrations all of the S-1 will be dissociated from actin yet it has always been observed that a small fraction of the S-1 or HMM binds to actin as the steady state

ATPase activity reaches Vmax. The results of Stein et al (1979) show that the binding between S-1 and actin does indeed occur during steady state ATP hydrolysis at high actin concentrations. They suggest this must be due to incomplete dissociation of the acto S-1 complex by ADP thus developing a rapid equilibrium between A-M*-T and M*-T. In addition Stein and co-workers did not find an inhibited ATPase activity at high actin concentrations, and therefore proposed that ATP hydrolysis could occur without dissociation of the acto S-1 complex (A-M-T>A-M-D-P_i), a step that was postulated not to occur in the three models already discussed. They concluded that the refractory state M-D-P_i(R) was not refractory to binding actin, but in effect bound to actin with the same binding constant as M-T. With this they proposed the model in Figure I(4). The kinetic model of Stein et al (1979) is similar to the original refractory state model in that the rate limiting transition occurs after the initial P_i burst. It differs from the original refractory state model in that M-D-P_i can form a complex with actin, therefore the initial P_i burst and the rate limiting transition from M**D-P_i to M-D-P_i can occur with the S-1 bound to actin as well as dissociated from actin. Although the refractory and non refractory state can bind weakly to actin the slow transition from the refractory state still limits the rate of which S-1 can undergo the subsequent rapid transitions to A-M*-D and A-M the states in which the S-1 is strongly bound to actin. Thus, rather than a cycle from the unattached to the attached states occuring as in the original refractory state model a cycle from weakly bound to strongly bound states occurs. Presumably one of the

strongly bound states is the major force producing state in the. muscle (Eisenberg and Hill, 1978) Although the crossbridge in the refractory state may be weakly attached to actin in a rapid equilibrium it is still blocked from transforming to the major force producing state until it undergoes a conformational change to the non refractory state.

Two additional aspects of this kinetic model are of interest with regard to the mechanism of crossbridge action in vivo. First this model has no step where the S-1 must detatch from actin. If during the ATPase cycle the S-1 attached to actin goes from 90° to 45° it can go back to 90° without first detaching from actin. If the weakly attached states are in rapid equilibrium with unattached states and can rapidly detach when they exert negative force a manditory detachment step may not be necessary (Eisenberg and Greene, 1980). Second, in the Lymn-Taylor model it was assumed that A-M*-T was a 45° state while A-M**-D-P_i was a 90° state. In the Stein model there is no manditory detachment step so it is unknown which attached states are 90° and 45° although they postulate that the weakly attached states are 90° while the strongly attached states are at 45°.

Complete Crossbridge Models

The first major attempt to combine the structural, biochemical, and physiological observations into a model of crossbridge action was undertaken by Eisenberg and Hill (1978). The biochemical basis for the crossbridge model was a simplified version of the actomyosin cycle introduced by Chock et al (1976). The physiological and structural basis for the model came from the studies of (Huxley,

1957; Huxley and Simmons, 1971; Hill, 1974; Hill, 1977).

The model consists of four states; two unattached states, the refractory and the non refractory and two attached states A-M-D-P_iN (90°) and A-M-D (45°) which occur in significant concentrations, while the other states are assumed to be transient intermediates. In their model Eisenberg and Hill utilize Huxley's 1957 variable "x" the measure of the active site relative to the crossbridge. The value of x is directly related to the angle of the attached crossbridge (ie at a 90° angle x=80 A°, at 45° angle x=0). In addition, the force energy studies of T.L. Hill (1974, 1977) indicate that the free energy of the attached state depends on x due to its elacticity, stretching or compressing the crossbridge increases the free energy level. The slope of the free energy curve for an attached state at any value of x is equal to the force exerted by the crossbridge state of the value of x.

The Eisenberg and Hill (1978) model proposes that the crossbridge initially in the refractory state transforms to the non refractory state and binds to actin to form the 90° attached state A-M-D somewhere near x=0, rapidly changing to state A-M-D in the 45° state. By incorporating the independent elastic element into the crossbridge as proposed by Huxley and Simmons (1971) they suggest that the change to the 45° state stretches considerably the elastic element in the S-2 of the crossbridge causing force development. The crossbridge simultaneously moves along x and drops in free energy as the spring in the S-2 shortens. When the spring reaches its equilibrium position or is slightly compressed the crossbridge releases ADP and P_i, rebinds ATP, and detaches from

the actin to form M-D (+D). It is then ready to begin a new cycle.

Several key features of this model should be emphasized. First the crossbridge does positive work in an attached state when it drops in free energy only if it simultaneously moves along x. Second, in the 90° and 45° states the crossbridges prefer being at 90° and 45° angles respectively but can rotate to higher angles where they exert positive force and lower angles where they exert negative force. Third and most important, the elastic properties of the crossbridge states do not determine the rate constants between states, therefore the rate constants between the 90° and 45° states do not depend on the thermal motion to stretch an elastic element as does the Huxley-Simmons model.

The fact that the rate constants between the 90° and 45° crossbridge states do not depend on the elasticity of the crossbridge allowed Eisenberg, Hill, and Chen (1980) to extend their 1978 model to include a free energy profile, four pairs of rate constants for the four states, and a proposal relating the biochemical parameters with the physiological parameters namely the ATPase rate and the force velocity curve.

Huxley (1957) proposed, and Eisenberg, Hill and Chen (1980) confirmed that many of the steady state properties of muscle could be explained by having the crossbridges attach at a moderate rate (f), detach very slowly (g) in the region where they exert positive force, and detach rapidly where they exert negative force. Where the attachment of the crossbridge is postulated to be relatively slow there is a marked decrease in the number of attached crossbridges that exert positive force as the velocity increases.

As in the H.E. Huxley (1957) model the decrease in force with increased velocity is primarily due to the slow attachment rate. Consequently the model predicts that the number of attached crossbridges will decrease as the velocity increases, therefore the Huxley proposal was a key factor in the rate constant values of the Eisenberg Hill and Chen model. In their model it is suggested in matching the high efficiency of muscle contraction (54%) that the binding constants of ATP and actin to myosin are similar. To make these binding constants alike requires an effective actin concentration of approximately 10 M. In addition by making the binding constants of ATP and actin to myosin similar the free energies of the 45° state and the refractory state are similar causing very little loss free energy when ATP dissociates the crossbridge from actin keeping the efficiency high.

The modified model of Eisenberg and Hill (1978) proposes that the crossbridge makes the transition from the refractory to the nonrefractory state ($M\text{-}D\text{-}P_R$ > $M\text{-}D\text{-}P_{iN}$). The rate constant for this transition is based upon the Vmax determined in biochemical experiments and is similar to "f" in the Huxley model. Following a rapid attachment step ($M\text{-}D\text{-}P_{iN}$ > $A\text{-}M\text{-}D\text{-}P_{iN}$) the crossbridge undergoes a transition from the 90° state ($A\text{-}M\text{-}D\text{-}P_{iN}$) to the 45° state (A-M-D). The rate constants for this transition were based on the rate of recovery of force in the isometric transient as a function of the amount of release of stretch (Eisenberg and Hill, 1978). The rate of detachment from actin was assumed to depend on the rate of ADP release with the subsequent rebinding of ATP and detachment of the crossbridge being rapid. The rate of ADP release

dependent on x was slow until the crossbridge reached minimum free energy at x=0, similar to the "x" dependence of the detachment rate constant "g" in the Huxley model. Following the detachment of the crossbridge the crossbridge again is in the refractory state M-D-P_{iR} ready to begin another cycle.

The models proposed by Eisenberg and Hill (1978) and Eisenberg Hill and Chen (1980) assumed that the crossbridge detached and reattached every cycle. The crossbridge model of Stein et al (1979) requires a mechanism for the cyclic detachment of the crossbridge in vivo as the muscle shortens although ATP hydrolysis can occur in vitro with or without detachment of S-1 from actin, and the large decrease in free energy associated with ATP hydrolysis.

Eisenberg and Green (1980) have revised their previous model to include the following modifications 1) based on the data of Marston et al (1978) they propose that ATP like its analogue AMP-PNP may increase the prefered angle of the crossbridge from 45° back to 90° 2) the minimum free energy of AMT is lower than M-T consistant with the marked free energy drop when ATP binds to actomyosin 3) their model suggests that the crossbridge in the refractory state rapidly attaches to actin in a region where it exerts positive force, the crossbridge then makes the rate limiting transition to the non refractory state. Following this transition the conformational change associated with P_i release occurs rapidly at about x=80 A° just as the conformational change induced by ATP changes the prefered angle of the crossbridge from 45° to 90°. On the other hand conformational changes associated with P_i and ADP release return the crossbridge to a preferred angle of 45°. Relating the

data of Marston et al who suggest that ADP lengthens the muscle fiber Eisenberg and Greene (1980) suggest that the release of P_i returns the preferred angle of the crossbridge to 50° while ADP release is necessary to return it all the way to the preferred angle of 45°. This ability of ADP to increase slightly the preferred angle of the crossbridge may provide a mechanism for keeping the crossbridge attached to actin until it has nearly completed its power stroke. Following the release of ADP the crossbridge rebinds ATP to form A-M-T with a preferred angle of 90° which exerts negative force breifly before it detaches and begins a new crossbridge cycle. This detachment occurs only when the muscle is shortening. In the isometric state the crossbridge will not detach as it hydrolyzes ATP, rather it will oscillate between the various attached states spending most of its time in state A-M-D exerting positive force.

In contrast to the model of Eisenberg Hill and Chen (1980) in the new model the number of attached crossbridges will probably not change as the shortening velocity increases because the refractory state is weakly attached to actin consistent with the Xray diffraction studies of Podolsky et al (1976).

Specific Kinetic Steps

Myosin

The pre-steady state kinetic studies (Tonomura and Inoue, 1975; Bagshaw et al, 1974; Taylor, 1977; Stein et al 1979) have presented evidence suggesting that either one or both of the myosin heads obey the mechanism in Figure I (4) for the binding and hydrolysis of ATP.

This mechanism has been incorporated into the upper line of the Stein et al, (1979) model and includes three steps, the binding of ATP to myosin, the hydrolytic step and the release of products. Using the nomenclature of Bagshaw et al (1974) * and ** qualitatively represent the amount of fluorecence enhancement shown by various intermediates.

The binding of ATP to myosin

The binding of ATP is postulated to occur in two steps the weak binding of ATP to form the collision intermediate M*-T, followed by an irreversible first order conformational change to form M*-T (Bagshaw and Trentham, 1974). This conformational change is considered irreversible because K-2 has been reported to be about 10^{-5} S^{-1} (Bagshaw and Trentham, 1973; Mannherz et al, 1974; Goody et al, 1977) much less than K_4 which is about 10^{-2} S^{-1} (Trentham et al, 1972). Thus the rate of detachment of ATP from myosin is much slower than the rapid binding of ATP to myosin. In addition, the binding constant of ATP to myosin has a value of about 10^{10}-10^{11} M^{-1} (Bagshaw et al, 1974; Johnson and Taylor, 1978; Chock et al; 1979), therefore a very large change in free energy occurs when ATP binds to myosin (Stein et al, 1979).

The hydrolysis step

Most workers agree that several phenomenon accompany the interaction of ATP with myosin. An enhancement of tryptophan fluorescence (Bagshaw et al, 1974) and ultraviolet absorbance occurs (Morita, 1969), H^+ is released (Chock and Eisenberg, 1974; Bagshaw and Trentham, 1974; Chock, 1979) and ATP is rapidly hydrolyzed in the initial P_i burst. Indirect measurements in early studies suggested that the binding of ATP (M*T) or the

ATP hydrolyzed in the intial P_i burst (0.8/mol myosin) at the same steady state rate.

The Release of Products

Following the initial P_i burst the model of Stein et al (1979) suggests that another conformational change occurs (K_7) the transition from the refractory to the non refractory state with the forward rate constant equal to the maximum rate of acto S-1 ATPase. This transition has been postulated to be the rate limiting step, although there is still some controversy as to whether it occurs when the S-1 is both attached to and detached from actin (Adelstein and Eisenberg, 1980). The next steps involved are the product release K_9 and K_{11}. Like the binding of ATP the release of ADP and P_i probably occurs in at least two steps. The release of P_i (K_9) involves a conformational change followed by the release of P_i from the M-D-P_i collision intermediate (Stein et al, 1979), while the release of ADP occurs with formation of a collision intermediate followed by two conformational changes (Adelstein and Eisenberg, 1980). The conformational change associated with P_i and ADP release is postulated to be very slow in the absence of actin and above a temperature of 5° C it is the rate limiting step in the myosin ATPase cycle (Webb et al, 1978) The value of K_9 the binding constant of P_i to myosin (a reversible step) is between 1 M^{-1} and 10 M^{-1} (Webb et al, 1978) with a significanct free energy change occuring when P_i is released. The binding constant of ADP to myosin is approximately 10^5 M^{-1} (Greene and Eisenberg, 1980).

The Actomyosin Cycle

S-1 binds to actin with a binding constant K_2 of about 10^7-10^8 M^{-1}, ADP weakens the binding about 30 fold, and ADP+P_i weakens the binding about 3000 fold (Greene and Eisenberg, 1980; Stein et al; 1979). All the myosin states can bind to actin although M-T, M-D-P_{iR} and M-D-P_{iN} bind quite weakly while M-D and M bind more strongly (Stein et al, 1979). The second order rate constant for the binding of M to actin is about $5x10^6$ M^{-1} S^{-1} for all of the myosin states. If this is the case it has been suggested by Adelstein and Eisenberg (1980) that the differences between the binding constants of the various myosin states to actin may reflect differences in their rate of detachment from actin. With this they propose that M-D-P_{ir} and M-T would rapidly detach from actin with rate constants about 10^3 S^{-1} explaining the fact that ATP dissociates the acto S-1 complex so rapidly (Lymn and Taylor, 1971).

The steps that occur when various myosin states bind to actin are included in the lower line of the Stein model (Figure 1 (4)). The binding of ATP to A-M (K_4) like the binding of ATP to myosin occurs in two steps, the formation of a collision intermediate followed by a conformational change. The ATP rapidly dissociates actomyosin (Lymn and Taylor, 1971) with a rate constant of 10^7 M^{-1} resulting in a large drop in free energy. The next step in the cycle (K_6) the hydrolysis of ATP on actomyosin (A-M-T > A-M-D-P_{iR}) may only occur at high actin concentrations since the binding of M-T and M-D-P_{iR} to actin is weak. Major evidence for the occurence of this step includes the data of Stein et al (1979)

who discovered no inhibition of ATPase activity at high actin concentrations and Sleep and Hutton (1978) who determined that ATP hydrolysis can occur without the dissociation of the actomyosin complex.

The rate limiting transition from A-M-D-P_{iR} to A-M-D-P_{iN} occurs at the same rate whether the S-1 is detached or attached to actin (Stein et al, 1979; Sleep and Hutton, 1978). A possible physical transition from the refractory to the non refractory state is the mechanism of ^{18}O exchange first proposed by Sartorelli et al (1966). They noted that when the Mg^{+2} dependent ATPase reaction proceeded in $H_2{}^{18})$ the product P_i incorporated about 3-^{18}O atoms. This was later confirmed by Bagshaw et al (1974) and Wolcott and Boyer (1974). Bagshaw et al (1974) determined that M-T exchanges about 75% of its four terminal O_2 within three seconds suggesting that not all of the terminal O_2 atoms in M*T and M**D-P_i have exchanged, while Wolcott and Boyer (1974) concluded that 3.16 of the 4 O_2 atoms are incorporated by H_2O.

Further studies of Bagshaw and Trentham (1974) and Boyer et al (1973) have shown that the dissociation of bound ATP to the free ATP pool is extremely slow and reversible ATP cleavage was suggested as the mechanism of intermediate O_2 exchange. Current evidence (Sleep and Boyer, 1978; Sleep and Hutton, 1978) is consistent with the ATP/HOH exchange arising from multiple reversal of the hydrolytic step of the ATPase pathway. An ATP molecule bound, one O_2 is incorporated into the P_i, and on cleavage upon reversal there are three chances out of four of this O_2 being in the y phosphate of ATP.

The release of this bound ATP into the medium results in the ATP/HOH exchange.

Schlucka and Levy (1977) have concluded that the P_i0 are non equivalent with respect to three O_2 exchanging fast and the fourth exchanging five times more slowly. In contrast, using $y\text{-}^{18}OATP$ and the formation of a valuable derivative of the product P_i for direct mass spectral analysis, Sleep et al (1978) have determined that the P_i0 are equivalent and the apparent non equivalencies arise from the contribution of a second ATPase pathway to the overall flux. Sleep and Boyer (1978) have suggested that $M\text{-}D\text{-}P_{iR}$ may possibly have a P_i bound to the enzymme in the metaphase form. Thus ATP cleavage could precede H_20 addition and H_20 would be added to the metaphosphate in the next step.

The final step of the actomyosin cycle involves P_i (K_{10}) and ADP (K_{12}) release from actomyosin. Stein et al (1979) have proposed that the forward rate constants of these steps are faster in the presence of actin than its absence accounting for the actin activation of the myosin ATPase activity. Although the rate of P_i release is postulated to be quite slow while the rate of ADP is fast (White, 1977). There appears to be a large free energy drop across the step involving release of P_i from $A\text{-}M\text{-}D\text{-}P_i$ while very little free energy change occurs with the release of ADP.

In summary in the myosin ATPase cycle the major decrease in free energy occurs during the ATP induced conformational change; the other steps being relatively close to equilibrium. The slowest step is the conformational change associated with P_i release with ADP release slow at very low temperatures. In the actomyosin cycle two

steps yield large decreases in free energy; the binding of ATP and the release of P_i. Lymn and Taylor (1971) predicted that P_i release was the rate limiting step while Stein et al (1979) suggest it is the transition from the refractory to the non-refractory state.

Relaxation

In vertebrate striated muscle, contraction is controlled by the effect of intracellular concentration of Ca^{+2} on the regulatory proteins troponin and tropomyosin which are integral components of the thin filament. At low Ca^{+2} concentrations the regulatory proteins inhibit the interaction of actin with the myosin crossbridges, at high concentrations this inhibition is released (Huxley, 1972)

The structural interpretation of this mechanism is based on a number of x-ray diffraction studies (Huxley, 1972; Squire, 1974; Hasselgrove, 1972) where observed changes in the second (increase) and third (decrease) layer lines from relaxed and contracting muscles were accounted for solely in terms of a change in the position of the tropomyosin molecule on the grooves of the actin helix. This model termed the steric blocking mechanism suggests an arrangement whereby the tropomyosin molecule occupies a position in the actin groove on or near the postulated myosin S-1 site of the actin molecule in the relaxed or "off" position. In this position the tropomyosin molecule may inhibit actin myosin interactions by physically blocking the active site for myosin attachment (Huxley, 1972; Hasselgrove, 1972). When the muscle is activated and Tn-C binds to Ca^{+2} a modification in the structure of the tropomyosin

molecule to which the Tn molecules are attached occurs so that it moves from a position of 45-50° in relaxed muscle very close to the HMM site to a position of orientation 65-70° in contracting muscle considerably further away from the attachment site (Squire 1974). In this way it would be possible for one Tn molecule to exert a controlling influence over all seven actin monomers in contact with the tropomyosin molecule controlled by one troponin complex.

Recently Seymour and O'Brien (1980) using three dimensional image reconstruction tecniques to examine the structure of muscle thin filaments still attached to the Z lines determined that tropomyosin consistently occupies positions on the opposite side of the actin groove to that of the postulated S-1 binding site. These results also confirmed by Taylor and Amos (1981) indicate that the tropomyosin in the inhibited state is located on the opposite side of the groove from the position required for the steric blocking model described above. Therefore this model had to be revised or a new model hypothesized in order to explain the inhibitory mechanism preventing the interaction of actin with the myosin crossbridges in the presence of low Ca^{+2} concentrations.

Taylor and Amos (1981) using three dimensional image reconstruction of electron micrographs have assigned actin to a new position in the thin filament structure leading to a different geometry of the myosin S-1 actin interaction. They have determined that the actin binding site is not in the middle of the S-1 fragment but is toward the outside. A portion of the S-1 extends into the long pitch helical groove where it appears to come into very close

contact with tropomyosin. Together with the results of Seymour and O'Brien (1980) who determined a reversal of the thin filament polarity Taylor and Amos (1981) have concluded that a revised steric blocking model can be reconstructed to conform to this new data.

In contrast, Chavlovich and Eisenberg (1982) have proposed a new model for the inhibited interaction of actin with myosin at low Ca^{+2} concentrations. They suggest that since the crossbridge normally exists with bound ATP or ADP-P_i (not bound ADP) in relaxed muscle (Taylor, 1979) the steric blocking model predicts that the troponin-tropomyosin system should inhibit the binding of A-1 ADP-P_i as well as S-1 ADP to regulate actin in the absence of Ca^{+2}.

Using stop flow turbidity measurements they have determined that in the absence of Ca^{+2} the binding constant of S-1 ATP or S-1 ADP-P_i to regulated actin was only decreased by 56% of the value in the presence of Ca^{+2} although the rate of ATP hydrolysis was decreased to 6% of the rate with Ca^{+2} present. This indicates that inhibition of the rate of ATP hydrolysis in the absence of Ca^{+2} is not the result of the binding of S-1 to regulated actin.

In addition they have also demonstrated that the removal of Ca^{+2} affects the rate of regulated actin activated S-1 ATPase activity primarily by lowering the maximal ATPase rate (Vmax) rather than the apparent binding constant of S-1 to actin (K-ATPase). They suggest that this data implies that in the absence of Ca^{+2} troponin-tropomyosin inhibits the ATPase activity by inhibiting a kinetic step in the cycle of ATP hydrolysis, with the most probable

step being the conformational change associated with P_i release in the cycle of ATP hydrolysis rather than the simple steric blocking model of muscle relaxation.

REGULATION OF CONTRACTILE FUNCTION

When determining the effect of various physiological parameters on the contractile apparatus, % tension vs Ca^{+2} data is utilized treated on the basis of the Hill equation: $P = P_0/(1+Q/[Ca^{+2}]^n)$ where p is isometric tension at partial activation, P_0 is isometric tension at full activation, Q is a constant, and n is the Hill value which distinguishes between independent ($n=1$) and non-independent binding of Ca^{+2} which may have positive ($n>1.0$) or negative cooperativity ($n<1.0$). In examining the regulatory features of the contractile system namely Mg^{+2}, $Mg^{+2}ATP$, ionic strength, ph, and phosphorylation three parameters of the length tension vs Ca^{+2} curve can be altered; 1) a change in maximum tension P_0 2) a change in Ca^{+2} sensitivity (the Ca^{+2} necessary for 1/2 max activation) 3) the cooperativity with respect to Ca^{+2} alterations that result in a changed slope of the activation curve (Rupp, 1980).

$Mg^{+2}ATP$

Classically $Mg^{+2}ATP$ is thought to perform two major functions in skeletal and cardiac muscle; it provides energy for the contractile process and acts as a plasticizing agent which maintains muscle extensibility (Weber and Murray, 1973). $Mg^{+2}ATP$ is the substrate of actomyosin ATPase, and by binding to the myosin moiety of the crossbridge promotes dissociation of the myosin head from actin. Under normal conditions ATP is in the range 3-6 mM (Burt et al, 1976), and is present as a complex with Mg^{+2} due to excess Mg^{+2}.

An alteration in $Mg^{+2}ATP$ concentration affects several

physiological parameters of muscle contraction. Maximum velocity of shortening depends in a roughly hyperbolic fashion on $Mg^{+2}ATP$ concentration while isometric tension displays a bell shaped dependence with $Mg^{+2}ATP$, and the force velocity relation becomes more curved with an increase in MgATP concentration (Ferenczi et al, 1981).

In addition $Mg^{+2}ATP$ has been shown to affect at least indirectly the activation process in skeletal muscle, that is the interaction of Ca^{+2} and the myofibrils that lead to the onset of contractile activity. The relationship between Ca^{+2} concentration and ATP hydrolysis for isolated myofibrils (Portzehl et al, 1969; Weber, 1969) and tension generation in skinned skeletal (Brandt et al, 1972; Godt, 1974) and cardiac muscle (Best et al, 1977) is dependent on $Mg^{+2}ATP$ concentration. As $Mg^{+2}ATP$ concentration is increased there is a decrease in maximum isometric tension the fibers can develop (Best et al, 1977; Orlentlicher et al, 1977), a shift in the pCA % max tension relationship in the direction of increased Ca^{+2} concentration required for activation and tension generation (Best et al, 1977; Fabiato and Fabiatio, 1975; Godt, 1974; Orlentlicher et al, 1977), an increase in the steepness of the pCa max tension relationship (Kerrick and Donaldson, 1975; Best et al, 1977) and an increase in ATPase activity (Portzehl et al, 1969).

Weber and Murray (1973) and Orlentlicher et al (1977) have proposed schemes which account for both the shift to the right of the % tension vs Ca^{+2} curve as $Mg^{+2}ATP$ increases and the decline of maximum tension at high substrate concentrations. At low $Mg^{+2}ATP$ concentrations (in the uM range), tension generation, actin-myosin interaction, and myofibril ATPase activity are no longer affected by

changes in Ca^{+2} concentration (Weber and Herz, 1963; Weber, 1969). To account for this Weber and Murray (1973) suggest the formation of rigor complexes at low $Mg^{+2}ATP$ concentrations which turn on the filaments in the absence of Ca^{+2}. Under normal circumstances the thin filament is in a special "on" state ordinarily achieved by the binding of Ca^{+2} to Tn before it can form an active complex. In the absence of Ca^{+2} the rigor complexes can be formed in the "off" as well as the "on" state. In the presence of Ca^{+2} the rigor complexes act to modify any actins that were already turned on. Weber and Murray (1973) termed this the "potentiated state" which they determined exhibits the greatest contractile activity. From this they further proposed a mechanism of cooperation among the molecules of the thin filament which together affect the Ca^{+2} affinity of the thin filament Tn molecules. Based upon the Weber-Murray study, Godt (1974) and Best et al (1977) have suggested that at physiological levels of $Mg^{+2}ATP$ there are practically no rigor complexes (on state) and contraction is activated only after Ca^{+2} ions bind to the high and low affinity sites of Tn. At much lower $Mg^{+2}ATP$ concentrations formation of the rigor complexes causes cooperative transfer of the low affinity sites to high affinity Tn site so that contraction is initiated at lower free Ca^{+2} concentrations Orlentlicher et al 1977) modified the Weber-Murray model by renaming and redifining the three states as follows;

Weber-Murray	Orlentlicher	Definition
off	ES_2	double bound nucleotide
on	ES	single bound nucleotide
potentiated	E	no nucleotide

According to their scheme as substrate concentration is increased the equilibrium among these states in the presence of Ca^{+2} shifts from rigor (all E) through a range of tension producing substrate concentrations to the totally relaxed conditon (all ES_2).

Calcium

It is now well established that myoplasmic free Ca^{+2} concentration regulates contractile activity in vertebrate skeletal and cardiac muscle. The mobilization of Ca^{+2} from extracellular and/or intracellular sites to the contractile proteins involves processes modulated by a complex interacting series of mechanisms that begin with excitation of the sarcolemma and end when Ca^{+2} binds to the Tn complex.

It has recently been documented in hearts from all species that force declines rapidly when Ca^{+2} is removed from the medium (Rich and Langer, 1975; Bailey and Long, 1974) in marked contrast to skeletal muscle where force persists for many minutes to several hours (Rich and Langer, 1975). This implies that a readily exchangeable component of Ca^{+2} is crucial to the maintenance of force in heart muscle but not skeletal muscle. It is well established that the E-C coupling sequence of cardiac muscle unlike skeletal muscle is dependent upon a source of extracellular Ca^{+2}. It has been suggested that this extracellular Ca^{+2} has a regulatory role in myocardial contraction although the quantity of Ca^{+2} from this source actively delivered to the myofilaments is uncertain.

Recent studies indicate that the regulatory extracellular Ca^{+2} is bound to the sarcolemmal membrane of cardiac cells (Bers and Langer,

1979; Philipson and Langer, 1979; Philipson et al, 1980). These studies indicate a quantitative relationship between total sarcolemmal bound Ca^{+2} and contractility of the rabbit ventricle although the mechanism of this regulation has not been elucidated. Studies on the isolated sarcolemma from the heart (Philipson and Langer, 1979) indicate that there are two classes of Ca^{+2} binding sites within the cell membrane. There are a relatively small number of sites with high affinity (K_m=20 uM) and an approximately ten fold increase in the number of sites with lower affinity (K_m=1.2 uM). It appears that the high affinity sites remain saturated at the usual levels of Ca^{+2} and play little role in the regulation of the contractile force. Evidence indicates however that the low affinity sites are important in the control of contractility (Philipson and Langer, 1979). If "triggered release" is operative in the intact skinned cell its force development would be modulated by the gradation of the amount of Ca^{+2} which crosses the sarcolemma. Therefore whether surface bound Ca^{+2} activates the myofilaments directly or serves as a trigger for subsequent sarcotubular release its magnitude determines the force of the twitch.

From the preceding discussion it has been determined that the degree of activation and consequently the amount of force can be regulated by the extent to which intracellular Ca^{+2} rises when the muscle cell has been stimulated. Once Ca^{+2} enters the interfilament space it affects several contractile parameters;

1) tension and maximum unloaded shortening (Vmax) are sensitive to changes in the Ca^{+2} concentration. As Ca^{+2} increases peak tension increases associated with a corresponding increase in

crossbridges attached to actin at one moment (Herzig and Ruegg, 1980; Honig and Takauji, 1976). With the Ca^{+2} induced increase in the number of crossbridges the ratio of contractile force to internal shortening resistance increases thereby facilitating shortening and an increase in its velocity. Herzig and Ruegg (1980) determined that Vmax is also altered independent of tension and stiffness indicating that the number of crossbridges interacting with actin filaments is not the only factor determing Vmax.

2) the isometric tension, actomyosin ATPase and immediate stiffness show a well known sigmoidal dependence on pCa with 1/2 maximal activation at approximately $7x10^{7}$ M. The Ca^{+2} required for the 1/2 maximal effect upon Vmax is $2x10^{-6}$ M (Herzig and Ruegg, 1980).

3) Allen and Blinks (1978) found that variations of the concentration of Ca^{+2} in the bathing medium alters the relation between length and tension of cardiac muscle. Further to this Fabiato and Fabiato (1975) determined that the process by which Ca^{+2} is translocated to activate the myofilaments is length dependent. An increase in sarcomere length above its optimum decreases Ca^{+2} release from the SR of skeletal muscle (Herzig and Ruegg, 1980) while Fabiato and Fabiato (1975) determined that Ca^{+2} triggered release of Ca^{+2} from the SR is strongly inhibited by a decrease in sarcomere length.

Phosphorylation

Phosphorylation and dephosphorylation of myofibrillar proteins has been considered a potentially important biochemical mechanism for regulating muscle contracion since the discovery that myosin and troponin purified from rabbit skeletal muscle were phosphoproteins and

that purified subunits of these two proteins could be phosphorylated by different protein kinases (Perrie et al, 1973; England et al, 1972). Although the properties of the phosphorylation reactions catalyzed by the protein kinases have been extensively investigated a functional role related to contraction for phosphorylation of specific myofibrillar proteins has only recently begun to emerge. Although the SR, sarcolemma, Tn-T, and tropomyosin also contain phosphorylation sites, this discussion will be limited to Tn-I and myosin.

As previously mentioned, the serine 20 residue located in the chain of twenty six additional amino acid residues was partially phosphorylated in the normal beating rabbit heart and the P motiety attached to the serine residue was virtually the only P group that exchanged with the intracellular P pool (Moir et al, 1980) leading to the conclusion that phosphorylation may be significant in regulating cardiac contractile activity.

Until 1976 it was postulated that phosphorylation could cause an increase in the interaction between myosin and actin either by increasing the maximal amount of interaction at saturating Ca^{+2} concentrations or by decreasing the amount of Ca^{+2} required for activation (Rubio et al, 1975). Different results have been obtained by others. Ray and England (1976) were the first to show that phosphorylation of cardiac Tn-I increased the concentration of Ca^{+2} required for activation of the myofibrillar ATPase activity suggesting that the affinity of cardiac Tn for Ca^{+2} was decreased. Their results were supported by later studies of Holroyde et al (1979), Solaro et al (1976) and Reddy and Wyborny (1979). Others have reported that the effects of phosphorylation on Ca^{+2} activated ATPase are

more complex. Bailin (1979) found that there was not a simple rightward shift of the curve but more of a downward shift in that at very low Ca^{+2} concentrations there was also a decrease in ATPase activity. Others have also reported a decrease in cardiac myofibril ATPase activity at Ca^{+2} concentrations sufficient to saturate the Tn system (Wyborny and Reddy, 1978; Yamamoto and Ohtsuki, 1982) although there is no general agreement that it is simply a decrease in the Ca^{+2} sensitivity of the myofibril system.

The mechanism of inhibition of the myofibril ATPase activity appears to be an alteration in the Ca^{+2} binding properties of Tn-C, although Stull and Buss (1977) found that Ca^{+2} binding to the isolated troponin-tropomysoin complex was unaffected by the level of phosphorylation of Tn-I. In contrast, Moir et al (1980) and Holroyde et al (1979; 1980) found that phophorylation of Tn-I reduces the amount of myofibrillar bound Ca^{+2} over the same pCa range that myofibril ATPase is activated by Ca^{+2}. Since not all of the sites of cardiac Tn-C are regulatory (Holroyde et al, 1980; Johnson et al, 1980), it has been suggested that Stull and Buss (1977) may have been unable to detect the effect of Tn-I phosphorylation on the relevant binding sites of the troponin complex. Robertson et al (1982) have determined that it is the low affinity regulatory site that is modulated by the phosphorylation state of the serine 20 residue of cardiac Tn-I.

Both Robertson et al (1982) and Moir et al (1980) suggest that phosphorylation may be responsible for the well known increase in the rate of cardiac relaxation during inotropic response to catecholamines. Since the rate of Ca^{+2} transport by cardiac SR has been shown to be elevated following phosphorylation by cAMP dependent protein kinase

linked regulatory system in vertebrate striated muscle. Further studies by Holroyde et al (1979) support the contention that myosin can bind significant amounts of Ca^{+2} during contraction when the Ca^{+2} concentration exceeds 10 uM. However the rate of dissociation of Ca^{+2} from the myosin LC is much slower than the time course of relaxation during a single twitch in muscle (Bagshaw and Reed, 1977). Therefore, if Ca^{+2} binding to myosin in cardiac muscle serves any physiological function it would probably be tonic in nature (over a series of single contractions).

Kardami and Gratzer (1982) have determined that the degree of light chain phosphorylation is correlated in heart muscle with the active tension developed, in accordance with the results of Kopp and Barany (1979). From this it has been suggested that light chain phosphorylation may be involved in the formation of non-covalent bonds between myosin and actin in live muscle. Accordingly the physiological role of LC phosphorylation could be to increase the rate of combination of the crossbridge with the actin filaments (Barany and Barany, 1980).

To determine the cause of the increase tension with phosphorylation several investigators have explored the possibilty that phosphorylation could alter the Ca^{+2} binding properties of P light chains from skeletal and cardiac muscle. Alexis and Gratzer (1978) using fluorescence measurements observed that phosphorylation of the P light chain from rabbit skeletal muscle appeared to decrease the affinity of the protein for Ca^{+2}. Contrary to this, direct measurements of Ca^{+2} binding properties of cardiac and skeletal muscle myosins have not substantiated these previous reports. No significant effect of phosphorylation on Ca^{+2} binding properties in

the absence or presence of Mg^{+2} was noted with rabbit skeletal muscle myosins (Holroyde et al, 1979; Kuwagama and Yagi, 1979). These results indicate that phosphorylation does not necessarily affect the Ca^{+2} binding properties of myosin. It has been concluded that if these two biochemical events, phosphorylation and Ca^{+2} binding have any important role they may be independent with regard to physiological functions in muscle.

In addition to Ca^{+2} binding there was no significant difference between ATPase activities of purified myosin in the phosphorylated and non phosphorylated forms when ATPase activity was measured in the presence of Ca^{+2}, Mg^{+2}, or K^{+} (Morgan et al, 1976), while Holroyde et al, (1979) determined that the phosphorylation of myosin light chains slightly enhances ATPase activity at greater than 1/2 maximal activating free Ca^{+2} concentrations, but recent preliminary reports have claimed that phosphorylated myosin decrease the K_m value of actin for activation of myosin ATPase activity with no significant effect on the Vmax values (Pemrick, 1980) independent of the regulatory proteins.

Because the Ca^{+2} affinity of Tn is decreased when myosin is deficient of the LC2 several investigators have proposed that the conformation of myosin in vertebrate striated muscle is a fine tuner of the Tn Ca^{+2} switch. This would serve to control the saturation of the metal binding sites and thereby control the nature of myosin interaction with the actin filaments.

In summary, although it appears that phosphorylation of cardiac and skeletal muscle light chains are of physiological importance no clear effect of light chain phosphorylation on myofibril function has

been shown. Further study is required to determine the exact nature of myosin phosphorylation on the various contractile parameters.

Magnesium

Mg^{+2} is present in a millimolar range in both cardiac and skeletal muscle (Polimeni and Page, 1973). This is significant in terms of understanding contraction in vivo since Mg^{+2} has been found to have a depressant action on standard measures of contraction when its concentration is increased in a millimolar range (Portzehl et al, 1969; Kerrick and Donaldson, 1972; Fabiato and Fabiato, 1975).

Recent experiments on skeletal muscle indicate that Mg^{+2} may be a critical modulator of tension generation in relation to prevailing Ca^{+2} concentrations. Preliminary studies indicate that an increased Mg^{+2} is accompanied by a decrease in submaximal tension generation in frog skeletal muscle fibers (Kerrick and Donaldson, 1972), and maximal Ca^{+2} activated tension in cardiac muscle (Donaldson et al, 1981). Further studies indicate that as Mg^{+2} concentration increase the pCa vs % tension curve shifts to the right and its slope declines in skeletal muscle fibers (Kerrick and Donaldson, 1975; Solaro and Shiner, 1976). This indicates 1) a higher concentration of Ca^{+2} is required for a given tension and 2) less change in tension for a given change in Ca^{+2} concentration which suggests that Mg^{+2} in the physiological range greatly influences the Ca^{+2} sensitivity of the tension generating apparatus of skeletal muscle (Donaldson and Kerrick, 1975; Kerrick and Donaldson, 1972; Rupp, 1980). Like skeletal muscle an increase in Mg^{+2} concentration causes a shift in the pCa tension curves in the

direction of higher activating Ca^{+2} concentrations but Mg^{+2} appears to have lesser or no effect on the steepness of the activating divalent cation tension relationship in cardiac muscle (Kerrick and Donaldson, 1975; Solaro and Shiner, 1976).

Portzehl et al (1969) and Solaro et al (1976) found that the pCa-ATPase relation was shifted in the direction of decreasing pCa (increasing Ca^{+2} concentration) when Mg^{+2} concentration was increased to the millimolar range, indicating that the Ca^{+2} required for activation differs greatly with changes in Mg^{+2} concentrations. Although the relationship between Ca^{+2} and Mg^{+2} concentration and % maximal tension under specific physiological conditions was quantitatively the same for both skinned frog muscle fibers and mechanically disrupted rat ventricular muscle fibers (Kerrick and Donaldson, 1975). Solaro and Shiner (1976) have observed that the effects of Mg^{+2} on myofibrillar ATPase are different for cardiac and skeletal muscle. Between 1 and 5 or 10 mM Mg^{+2} the effect of Mg^{+2} is more pronounced in cardiac than skeletal myofibrils, and above 10^{-6} M free Ca^{+2} an increase from 0.04 to 1.0 mM Mg^{+2} enhanced cardiac and inhibited skeletal myofibril ATPase activity. At concentrations above 10^{-5} M free Ca^{+2} the cardiac myofibril ATPase activity is the most depressed. In contrast at all Ca^{+2} concentrations studied as free Mg^{+2} concentration was increased myofibril ATPase activity decreased at 0.04, 1.0 and 10mM of Mg^{+2} in skeletal muscle in agreement with the results of Weber (1959). The shifting and change in the shape of the pCa tension and ATPase curve as a function of the concentration of Mg^{+2} may be due to an effect on the affinity and/or degree of

interaction of the divalent ions with the Ca^{+2} binding sites. This was first proposed by Solaro and Shiner (1976) who determined that an increase in free Mg^{+2} from 1-10 mM caused a slight decrease in Ca^{+2} binding to the skeletal myofibrils but enhanced Ca^{+2} binding to the cardiac myofibrils.

It is well established that skeletal Tn-C contains four Ca^{+2} binding sites (Potter et al, 1977; Potter and Gergley, 1975). Two of these sites have a high affinity for Ca^{+2} (K_{Ca}= $5x10^8$ M^{-1}) and also bind Mg^{+2} competitively (K_{mg}= $5x10^4$ M^{-1}). These sites are termed the $Ca^{+2}Mg^{+2}$ sites and appear to function in stabilizing the troponin complex (Potter et al, 1981). The two other sites have a low affinity for Ca^{+2} (K_{Ca}= $5x10^6$ M^{-1}) are called the Ca^{+2} specific sites (Johnson et al, 1978) and are directly involved in Ca^{+2} regulation in muscle. These results have been confirmed by numerous studies (Johnson et al, 1979; Levine et al, 1978). Cardiac muscle differs from skeletal muscle in that it contains three Ca^{+2} sites, one low affinity and two high affinity with similar rate constants to skeletal muscle (Holroyde et al, 1980; Johnson et al, 1978). Ca^{+2} must occupy both of the skeletal Ca^{+2} specific sites or the one cardiac muscle site for activation to occur (Potter and Gergley, 1975).

Early studies suggested that the increased Ca^{+2} concentration required to activate tension development or myofibril ATPase activity at high Mg^{+2} concentrations influenced the ratio of Ca^{+2} to Mg^{+2} bound to the high affinity sites. In addition they assumed that Mg^{+2} at high concentrations bound also to the low affinity sites and would be ineffective for triggering contraction (Rupp, 1980).

Further studies by Potter et al, (1981) indicate that Mg^{+2} can not bind directly to the Ca^{+2} specific sites, thereby increasing the Ca^{+2} concentration required to produce contraction. These results may account in part for the previous shifts in the Ca^{+2} activation of tension observed at high Mg^{+2} concentrations.

Myosin

In addition to Tn Ca^{+2} binding, Mg^{+2} concentration may influence the Ca^{+2} binding properties of myosin. Solaro and Shiner (1976) found that an increase in free Mg^{+2} consistently depressed both skeletal and cardiac myosin bound Ca^{+2}. Potter (1975) determined that myosin binds 2 mol Ca^{+2} with an affinity of approximately $5x10^6$ M^{-1}, yet in the presence of 0.3 mM Mg^{+2} myosin binds two mol of Ca^{+2} with lower affinity ($5x10^5$ M^{-1}). Additional studies by Bremel and Weber (1972) indicate that an increase in Mg^{+2} from 3 uM to 1mM raises the Ca^{+2} required for the binding of the first Ca^{+2} by a factor of 10^3 and for the second ion by a factor of 10, indicating that Mg^{+2} competes for Ca^{+2} sites on myosin. Ca^{+2} binding to myosin is of particular interest in view of a possible Ca^{+2} regulatory system on the thick filament (Lehman, 1978). Although Ca^{+2} dissociation is too slow to occur during a single muscle twitch (Bagshaw, 1977), Ca^{+2} binding to myosin could be involved in long term modulation of the mechanochemical activity of muscle (Rupp, 1980).

The Sarcoplasmic Reticulum

Evidence has been cited that Mg^{+2} affects the release of Ca^{+2} into the cytoplasm from storage sites in the sarcotubules.

Podolosky (1975) observed an inhibition of quick contraction usually elicited by perfusion of the skinned fiber with Ca^{+2}. They concluded that Ca^{+2} stimulated release of Ca^{+2} from the sarcotubular Ca^{+2} stores could take place in the presence of high Mg^{+2} concentrations but the propogation of the sarcotubular Ca^{+2} release process throughout the cell was inhibited by the high Mg^{+2} concentration. In addition Ford and Podolsky (1972) speculated that an increased Mg^{+2} concentration may affect the rate at which Ca^{+2} is taken up by the sarcotubules. Fabiato and Fabiato (1975) confirmed this when they found that an increase in free Mg^{+2} increases the capacity and rate of binding for Ca^{+2} by the SR which is similar in both cardiac and skeletal muscle. Ebashi and Lipman (1962) observed that SR obtained from fragments of skeletal muscle had an increased capacity for Ca^{+2} at high Mg^{+2} concentrations while Tonomura (1972) found an increased rate of Ca^{+2} binding.

Other Effects of Magnesium

At 6° C the rate limiting step for the hydrolysis of ATP by myosin is the dissociation of ADP from the active sites (Taylor, 1972; Taylor et al, 1970; Bagshaw et al, 1973), a step which is sensitive to Mg^{+2}. The divalent Ca^{+2} ions increase the affinity of ADP for the enzymatically active portion of the myosin molecule, therefore it has been suggested that Mg^{+2} serves as a bridge linking the hydrolytically active portion of the myosin molecule and the nucleotide phosphates (ATP or ADP) together in a ternary complex (enzyme-metal-substrate) (Trentham et al, 1972).

Finally Mg^{+2} is involved in the polymerization of G-actin to F-actin. G-actin contains both a bound nucleotide (ATP) and a bound

divalent ion usually Ca^{+2} or Mg^{+2}. Both the nucleotide and the cation bound to G actin are freely exchangeable although removal of either reduces the stability of G-actin and it rapidly loses its ability to polymerize (Katz, 1977). Maruyama (1981) has observed at low ionic strength that Ca^{+2} retards polymerization of actin whereas Mg^{+2} accelerates it, concluding that the divalent metals probably play a role in the growth of the actin strand.

pH

Intracellular acidosis has a direct pronounced negative ionotropic effect on cardiac muscle and may be responsible for the reduced cardiac function in both ischemia and heart failure (Williamson et al, 1976). Since a greater fall in myocardial contractility is consequent upon an acidosis resulting from an increase in PCO_2 (respiratory acidosis) than from a decrease in bicarbonate (HCO_3^-, metabolic acidosis) (Pannier and Leuson, 1968) it has been postulated that intracellular pH is the important determinant of the effect of an acidosis on cardiac function.

There is general agreement about the events leading up to the development of intracellular acidosis; coronary occlusion, myocardial anoxia, and lactic acid production (Katz and Hecht, 1969). The ensuing intracellular acidosis results in an early rapid decline in force generation (Poole-Wilson and Langer, 1975; Donaldson and Hermanson, 1978; Donaldson et al, 1981), left ventricular developed pressure (Jacobus et al, 1982) and a decrease in maximum Ca^{+2} activated cardiac ATPase with little change in basal activity (Kentish and Naylor, 1977; 1979; Portzehl et al, 1969; Williams et al, 1973).

Although the events leading up to general acidosis and the resultant changes in contractility are well documented, the step linking the acidosis to impaired contractilty remain unsettled. There has been good progress in determining how acidosis might affect contractile activation and most data relates to the three stages in E-C coupling; the Ca^{+2} entry via the slow inward current, the release of Ca^{+2} from the SR, and the Ca^{+2}-Tn interaction.

Entry via the Slow Inward Current

One mechanism of Ca^{+2} induced Ca^{+2} release is Ca^{+2} entry via a slow inward current following depolarization. The amount of Ca^{+2} influx is probably sufficient to supply a fraction of the total activator Ca^{+2}. Williamson et al, (1976) has suggested that this leads naturally to the question of whether or not the slow inward Ca^{+2} is altered by conditions of acidosis. Chesnois et al (1975) have reported a decrease in the slow inward Ca^{+2} current in acid Ringer. In addition McDonald and McLeod (1973) and Schneider and Sperelakis (1974) have found that metabolic inhibition can block electrical activity which is presumed to be dependent on the slow Ca^{+2} current.

In contrast Poole-Wilson and Langer (1975) determined that intracellular acidosis produced a prolongation of the action potential or no change in the plateau of the action potential, while twitch tension fell more than 50%. Under the conditions of their experiments a large reduction in Ca^{+2} current seems unlikely since this would lower and shorten the action potential. Thus it appears possible to dissociate changes in slow Ca^{+2} current from the decline in contractility, with Poole-Wilson and Langer (1975) suggesting that

the mechanism of the negative ionotropic effect of acidosis is intracellular

Ca^{+2} Release from the SR

Cardiac and skeletal muscle respond to acidosis in different ways 1) in cardiac muscle ATPase activity decreases while in skeletal muscle it remains constant (Kentish and Naylor, 1979) 2) the Ca^{+2} requirement for 1/2 maximal activation is much greater in cardiac vs skeletal muscle indicating that Ca^{+2} sensitivity is more pronounced in cardiac muscle in an acid environment (Fabiato and Fabiato, 1978) 3) tension is diminished in cardiac myofibrils when pH is decreased from 7.0-6.5 while there is an enhancement of soleus twitch and tetanic tensions in response to a decrease in pH (Pannier and Weyne, 1970). It has been concluded that the ionotropic effects of varying pH on skeletal muscle is much less pronounced than those observed in cardiac muscle (Pannier and Leuson, 1968; Pannier and Weyne, 1970). Fabiato and Fabiato (1978) conclude that the effect of acidosis on the cardiac SR accentuates the already depressive action of acidosis on the myofilaments, while moderate acidosis in skeletal muscle the SR may in contrast compensate for the action on the myofibrils which is less pronounced than in cardiac muscle. This was explained when they determined that the amount of Ca^{+2} released from the SR of cardiac muscles is reduced even by moderate acidosis (Poole-Wilson and Langer, 1975; Fabiato and Fabiato, 1978) much less than the amount needed to activate the myofilaments completely therefore the Ca^{+2} released from the SR produces low levels of activation. In contrast, a small degree of acidosis increases the Ca^{+2} content of the SR of skeletal muscle (Nakumuru and Schwartz, 1970; 1972) and a larger amount of

Ca^{+2} is released compensating for any decrease in the sensitivity of the myofilaments to Ca^{+2}.

Calcium-Troponin Interaction

Since Ca^{+2} activation depends on binding of this ion one would expect that changes in pH would also influence the Ca^{+2} affinity of Tn-C. Katz and Hecht (1969) were the first to suggest that the cardiodepressant action of acidosis could be explained if protons compete with cytosolic Ca^{+2} for the Ca^{+2} binding sites on cardiac Tn-C. Concrete evidence for this proposal comes from studies on the Ca^{+2} dependence of tension and ATPase activity. The most dramatic effect of an increased hydrogen ion concentration is a shift in the PCa-ATPase relationship for both cardiac (Kentish and Naylor, 1979; 1977; Williams et al, 1975) and skeletal muscle (Portzehl et al, 1969) to a lower PCa value showing a depressed sensitivity of the sigmoidal myofibril ATPase relation. Fabiato and Fabiatio (1978) and Donalson and Hermanson (1978) reported that a decrease in pH substantially increases the Ca^{+2} required for 50% max tension development in cardiac and skeletal muscle with the effect somewhat larger in cardiac than skeletal myofibrils. Addition of saturating Ca^{+2} concentrations can overcome the depressed actomyosin ATPase activity (Williams et al, 1975; Serur et al, 1976). The literature supporting the Katz-Hecht (1969) proposal is varied. Fuchs and co-workers (1970) have demonstrated that lowering the pH decreases both the affinity of Tn-C for Ca^{+2} and the stability of the Ca^{+2}-Tn complex. Robertson et al (1978) have provided preliminary evidence based on indirect methods that indicates that H^{+} competes with Ca^{+2} for binding at the Ca^{+2} specific sites of Tn but not the

Ca^{+2} Mg^{+2} sites. Stull and Buss (1978) have recently published a detailed report of the effect of pH on Ca^{+2} binding to skeletal and cardiac Tn-C. Their work is of interest in that they used three different binding methods namely 1) gel filtration in the absence of Ca^{+2} buffer 2) equilibrium dialysis with EDTA as the Ca^{+2} buffer 3) equilibrium dialysis with EGTA as the Ca^{+2} buffer. With methods 1 and 2 they found no effect of pH on Ca^{+2} binding whereas with method 3 the binding curve was shifted to the right with a decrease in pH.

Although they could not explain the EGTA results they concluded that a decreased ph has no effect on the affinity or capacity of cardiac or skeletal Tn for Ca^{+2} in agreement with Fuchs (1974, 1979) and Potter and Gergley (1975). In addition Fabiato and Fabiato (1978) concluded that the force pCa curves obtained from skinned cells of acardiac and skeletal cells do not suggest a simple competion between H^{+} and Ca^{+2} for a single class of sites on Tn-C.

Ionic Strength

An increase or decrease in ionic strength alters several physiological features of cardiac and skeletal muscle. Resting tension and total force has been found to vary inversely with ionic strength in the range of 0.09-0.18 M KCl (Gordon et al, 1973; Thames et al, 1974; Gulati and Podolsky, 1978; Julian and Moss, 1981; April et al, 1968; Hamsher et al, 1974; Solaro et al, 1976; Gordon and Godt, 1969) while the Vmax and force-velocity relation are virtually unaffected by changes in ionic strength in cardiac (Honig and Takajui, 1976) and skinned skeletal fibers (Julian and Moss, 1981; Gulati and Podolsky, 1978).

The fact that tension generation decreases when ionic strength is increased might be brought about by 1) a decrease in the number of crossbridges attached to actin 2) a decrease in the tension generation capacity of each attached crossbridge with no change in the total number of attached (Julian and Moss, 1981) 3) the conductance of information from Ca^{+2} saturated Tn to actin may be inhibited when ionic strength is increased therefore at high KCl concentrations fewer than seven actin molecules may be switched on when Ca^{+2} binds and the availability of myosin sites are reduced (Thames et al, 1974) 4) Moos (1972) suggests the possibility that an increase in ionic strength decreases the electrostatic interaction of charged sites on actin and myosin reflected in the K^{app} with no effect on maximal ATPase rate at infinite actin Vmax 5) changes in intracellular ionic strength may alter the amount of Ca^{+2} released from the SR during stimulation since Kasai and Miyamoto (1976) indicate that skeletal muscle SR is stimulated to release Ca^{+2} when the ionic strength is lowered.

In addition, the major decline in tension seems to be accounted for by the effect of salt concentration on the contractile proteins relating to the tension produced by maximal Ca^{+2} activation. Several studies (Solaro et al, 1976; Gordon et al, 1973; Weber and Herz, 1963; Katz, 1968; Potzehl et al, 1969) indicate that as the ionic strength increases the Ca^{+2} sensitivity shifts so that more Ca^{+2} is required to attain a given % of maximal activity. From this it follows that ATPase activity will decrease when ionic strength increases (Goodno et al, 1978; Moos, 1972; Portzehl et al, 1969; Solaro et al, 1976; Warren et al, 1966).

The altered Ca^{+2}-ATPase relation and the decrease in ATPase activity may be related to changes in Ca^{+2} binding to Tn-C. Fuchs (1974), showed Tn-C binding to be independent of ionic strenth while Morimoto and Harrington (1973) demonstrated that myosin Ca^{+2} binding was slightly affected by changes in KCl concentration. Using the whole myofibril preperation Solaro et al (1976) determined that myofibril Ca^{+2} binding decreases when ionic strength increases. Since it could not be attributed to Tn-C or myosin binding they suggest that the myofibril Ca^{+2} control may vary with length changes, speed of shortening, and Ca^{+2} saturation of Tn-C all which involve changes in the number of connected crossbridges.

CARDIOVASCULAR ADAPTATIONS WITH TRAINING

Chronic moderate physical activity in animal studies results in enhanced cardiac performance. This includes increased cardiac output (Penpargkul and Scheuer, 1970; Guisti et al, 1978; Scheuer et al, 1974; Crews and Aldinger, 1967), stroke volume (Schaible and Scheuer, 1979), cardiac work (Penpargkul and Scheuer, 1970), oxygen consumption (Guisti et al, 1978; Scheuer et al, 1974), coronary blood flow (Guisti et al, 1978; Scheuer et al, 1974), and a decreased heart rate (Dowell et al, 1977; Whitehorn and Grimmenga, 1956; Crews and Aldinger, 1967; Schaible and Scheuer, 1979) after chronic programs of swimming (Guisti et al, 1978; Scheuer et al, 1974; Whitehorn and Grimminga, 1956; Penpargkul and Scheuer, 1970; Schaible and Scheuer, 1979) and running (Dowell, 1976; Schaible and Scheuer, 1979).

Changes in energy utilizing mechanisms have also been examined following physical training. Studies on mitochondrial respiratory activities in cardiac muscle from swimming or running exercised animals indicate no increase over sedentary controls (Holloszy, 1976; Oscai et al, 1971a; Scheuer et al, 1974; Baldwin et al, 1975) although they are markedly increased in skeletal muscle (Holloszy, 1967; 1976; Oscai et al, 1971b; Sordhal et al, 1977; Baldwin et al, 1975). Dowell et al (1977) suggests that this phenomenon may be attributed to more efficient energy utilization of electron transport generated energy in cardiac muscle and/or the presence of fewer Ca^{+2} transport sites which tend to utilize electron transport generated energy more efficiently. This is supported by

Penpargkul and Scheuer, (1970) who found that although mitochondrial respiratory enzymes did not increase, the aerobic/anaerobic energy ratio was greater in trained animals. Hearts of conditioned animals have greater endogenous glycogen stores (Scheuer et al, 1974), aldolase activity (Hearne and Waino, 1957), and pyruvate kinase activities with decreases in endogenous lipids and an increased turnover of free fatty acids (Scheuer et al, 1974). There is no marked change in the ATPase and/or CPK activities (Rawlison and Gould, 1959).

Two major changes in response to endurance exercise identified thus far in cardiac muscle involve varying degrees of morphological changes (heart weight and body weight) and ATPase activity in the contractile proteins. Both running (Penpargkul et al, 1980a; Resink et al; 1981; Baldwin and Terjung, 1975) and swimming programs (Bhan and Scheuer, 1972; Malhotra et al, 1976; Penpargkul and Scheuer, 1970; Bershon and Scheuer, 1977; Oscai et al, 1971a) indicate either no change or a decrease in heart weight (HW), a decreased body weight (BW), and an increased heart weight to body weight ratio (HW/BW) in male rats. Conversely female rats made to swim (Oscai et al, 1971a) or run (Baldwin et al, 1977) show no change in BW, with increased HW and HW/BW ratios. Thus the decreased body weight of the conditioned male rat is responsible for the increased HW/BW ratio, while the absolute increase in heart weight with no change in body weight results in the increased HW/BW ratio in females. When exercised male rats are compared with pair weighted sedentary animals, the hearts of trained rats are heavier than the pair weighted controls. This has been attributed to the food restriction in the control rats (Oscai, 1971a; Penpargkul et al 1980a). It has

been observed that enlargement of the coronary tree occurs in swim trained rats (Stevenson et al, 1964; Tepperman and Pearlman, 1961) while treadmill running has no effect on the collateral vessel development (Cohen et al, 1978).

Swimming programs have shown significant increases in cardiac myofibril (Hearne and Gollnick, 1961; Rupp, 1981), myosin (Bhan and Scheuer, 1975; Penpargkul et al, 1980a; Wilkerson and Evonuk, 1971; Medugorac, 1975), and actomyosin (Guisti et al, 1978; Penpargkul et al, 1980a; Bhan and Scheuer, 1972; Malhotra et al, 1976; Medugorac, 1975) ATPase activity. In contrast, treadmill exercise produces slight changes (Baldwin et al, 1977; Resink et al, 1981; Penpargkul et al, 1980a) or no change (Dowell et al, 1977; Tibbits et al, 1978; Baldwin et al, 1975; Penpargkul et al, 1980a) in myofibril, myosin, and actomyosin ATPase activity.

The intensity and duration of the exercise program relates to the ATPase changes incurred. Time studies have demonstrated that during the first eight weeks of a program the ATPase activity increases in proportion to the duration and severity of the program (Bhan and Scheuer, 1972; Wilkerson and Evonuk, 1971; Baldwin et al, 1977), and following two weeks of deconditioning the ATPase activity from hearts of previously conditioned animals is similar to that of sedentary controls (Guisti et al, 1978; Malhotra et al, 1976). The type of exercise program employed also corresponds to the ATPase changes found. Both moderate and intense swimming programs lead to significant increases in ATPase activity while only the very intense bouts of treadmill exercise show slight increases in ATPase activity (Baldwin et al, 1977; Resink et al, 1981; Penpargkul et al, 1980a) of the contractile proteins. This indicates that the cellular

adaptations of cardiac muscle to exercise is related to the type and amount of exercise performed, and that very distinctive differences may be apparent concerning the stress that running and swimming place on the heart muscle.

Most studies examining the causitive relationship between ATPase changes in cardiac tissue and training concern either an alteration in the myosin molecule or in light chain phosphorylation. In 1975 Bhan and Scheuer found an increase in the HMM ATPase activity of exercised animals indicating that the change was occuring in the head region of the myosin molecule. This change was not related to the oxidation of sulphydryl groups (Bhan and Scheuer, 1975; Resink et al, 1981) but the differential effect of ethylene glycol on activating myosin ATPase suggests that a local conformational change may occur in the myosin molecule from hearts of conditioned rats (Bhan and Scheuer, 1975). Rupp (1981) supported this contention when he determined that a three band pattern of myosin consisting of V_1 V_2 and V_3 with isoenzyme V_1 predominating in control tissue is changed into a single band identical to V_1 with slight traces of V_2 and V_3 detected following a twelve week swimming program. This indicates that a local conformational change does occur in conditioned rats through isoenzyme pattern alterations.

The ATPase changes following physical training are also accompanied by light chain alterations, with increases in the amount of LC 1 present from the myosin of swim trained rats (Medugorac, 1975). Further to this Resink et al (1981) found that the maximum

extent of phosphate incorporation is greater in hearts from running trained animals as is the phosphate content and the susceptibility to phosphorylation in response to an elevated extracellular calcium concentration. Their results suggest that an enhanced capacity for transarcolemmal Ca^{+2} flux in the hearts of trained animals may be responsible for enhanced Ca^{+2} dependent phosphorylation of myosin P light chains thus improving cardiac function.

Concomitant with the myofibril, myosin, and actomyosin ATPase changes are corresponding alterations in the contractility of the heart. Measurement of these enzymatic activities are elevated in states of increased contractility and diminished in states of decreased contractility (Hjalmarson et al, 1970; Goodkind et al, 1969). Thus it has been postulated that the reported exercise induced increase in cardiac contractile protein ATPase activity following training would seem to represent a cellular mechanism to account for the improvement in cardiac contractile function.

Conditioning programs have shown an increase in maximum velocity of superprecipitation of protein (Vmax) (Bhan and Scheuer, 1972; Resink et al, 1981), max dp/dt (Dowell et al, 1977; Penpargkul and Scheuer, 1970; Schaible and Scheuer, 1979), negative dp/dt (Guisti et al, 1978; Bersohn and Scheuer, 1977), tension output (Tibbits et al, 1978), tension development (Schaible and Scheuer, 1979), tension at a given diastolic length (Whitehorn and Grimmenga, 1956; Crews and Aldinger, 1957), and an increased preload and afterload (Scheuer et al, 1974). In additon there is an increased work capacity (Whitehorn and Grimmenga, 1956), reserve capacity

(Penpargkul and Scheuer, 1970), ejection fraction (Schaible and Scheuer, 1979), and peak systolic pressure. Maximum rate of shortening and maximum positive dp/dt are also greater in rats conditioned by swimming when compared to those conditioned by running. The increased rate of relaxation found in hearts trained by swimming correlates with the Ca^{+2} binding and uptake by the SR observed by Penpargkul et al (1980a). They consistently found a greater Ca^{+2} binding and uptake from the hearts of swimmers when compared to the sedentary controls (Penpargkul, 1979) with no changes apparent in the SR of hearts from runners (Penpargkul et al, 1980a; Dowell et al, 1977). This is consistent with the observed mechanical differences between the two exercise models (Penpargkul et al, 1980a).

The trend for swim trained animals to develop greater pressure, ATPase activity, and rates of relaxation than runners suggest that adaptations to swimming and running may be quantitatively different (Schaible and Scheuer, 1979). According to Flaim et al (1979), there are distinct differences between aquatic exercise and treadmill running during acute exercise bouts. Heart rate, cardiac output, and right and left ventricular coronary blood flow increase significantly during treadmill exercise while no change in these parameters were noted during aquatic exercise (Flaim et al, 1979). Stroke volume does not change in either group. Strenuous exercise by rodents elicits an O_2 consumption value of approximately 85 ml/kg/min during treadmill exercise with a much lower value of 60 ml/kg/min found during swimming exercise (McCardle, 1967; Shephard

and Gollnick, 1976). Water immersion had been considered to play a role in the biochemical changes found with swim trained animals that does not occur in the hearts of treadmill trained animals until refuted by Penpargkul et al (1980a). From the available data it is unclear why swimming and running should have different mechanical and biochemical effects on the heart.

DIABETES

Animal Models

Diabetes can be defined in animal and in man as a disorder associated with a number of clinical, physiological, and biochemical signs varyingly combined always with a degree of hyperglycemia (Cameron et al, 1972).

In recent years many studies have been conducted in animals since they provide a multitude of interacting factors contributing to the syndrome of diabetes that is not feasible in the afflicted human (Nakhooda et al, 1977). Several advantages have been provided by animal studies; 1) In humans genetic factors are difficult to study and control, while animal colonies allow many generations to be studied in a relatively short period of time 2) when dealing with animals entire muscles and/or organs can be excised and assayed, while this information is generally limited in humans 3) animals allow researchers to study diabetic pathology from the acute stages of the illness to the chronic since the life span of most animals is much shorter than in man (Mordes and Rossini, 1981). Although it is unlikely that any one model of animal diabetes will provide the ideal model for human disease that is identical in most respects, the advantages afforded by animals make them valuable research tools (Cameron et al, 1972).

Two models of diabetes are utilized in animal research; spontaneous and experimental diabetes. Spontaneous diabetes in animals is a common occurence which has been characterized in several species. Various strains include the diabetic mouse

(db/db), Chinese hamster, sand rat, spiny mouse, BB rat, celebese ape, and keeshod dog (Mordes and Rossini, 1981). The most prominent feature of spontaneous diabetes syndromes in these animals are hyperglycemia, transient hyperinsulinemia, and obesity (with the exception of the spontaneously diabetic Wistar rat (BB) and the Chineese hamster). The acute syndrome of the BB rat is metabolically similar to that observed in human subjects with juvenile onset diabetes (Mordes and Rossini, 1981).

In addition, many experimental tecniques are available for the creation of diabetic syndromes. These include the use of contrainsulin hormones such as glucagon, glucocorticoids, and growth hormone which have antagonistic effects on insulin, hypothalamic diabetes, virus induced diabetes, and toxic diabetes (Mordes and Rossini, 1981).

Toxic diabetes, the use of chemical agents to produce diabetes permits detailed study of the biochemical, hormonal, and morphological events that occur before and after the induction of the diabetic state (Mordes and Rossini, 1981). Two agents which have been most extensively studied are streptozotocin and alloxan.

Both streptozotocin and alloxan are beta cytoxins which produce permanent diabetes by destruction of the beta cells with a resulting insulin deficiency (Rerup, 1970). Both are diabetogenic in the rat, dog, hamster, monkey, and mouse with the effect being both strain and sex dependent (Mordes and Rossini, 1981; Rerup, 1970).

Several pronounced differences exist between the two drugs 1) alloxan has a circulatory half-life of one minute while

streptozotocin has a half-life of ten to fifteen minutes (Dubin and Soret, 1977; Ganda et al, 1976) 2) alloxan appears to act extracellularly on the beta cell membrane while streptozotocin acts intracellularly, therefore it appears that the two drugs damage the beta cell through two different mechanisms (Rerup, 1970) 3) streptozotocin decreases tissue NAD levels by decreasing synthesis and increasing its breakdown, while alloxan does not have this effect (Ganda et al, 1976; Dubin and Soret, 1977) 4) with alloxan diabetes, there is an ability of certain sugars to protect the animal against its effects when administered before the alloxan. These include glucose, mannose, and fructose, while 3-0 methyl glucose is the only substance that protects equally well against both.

Several studies (Malhotra et al, 1981; Penpargkul et al, 1980b) have administered 3-0 methyl glucose before streptozotocin as a control measure to determine whether the streptozotocin or the diabetic state itself is producing the physiological, metabolic, and biochemical changes associated with this model. In both studies the 3-0 glucose group had normal blood sugar values and the changes found in the diabetic group were not apparent in the 3-0 glucose group. Therefore it appears that streptozotocin injection alone is not sufficient to cause the deliterous effects of diabetes on the heart (this topic will be discussed in more detail in further sections)

Cardiomyopathy

Diabetes Mellitus has been associated with an increased mortality rate due to congestive heart failure (Kannel, 1978)

Recent post mortem studies have questioned the significance of coronary artery disease (atherosclerosis) as accounting for premature deaths in diabetes. Two seperate studies found no greater degree of obstructive disease in the coronary arteries of diabetics compared to control subjects (Vihert et al, 1969; Ledet, 1968). Epidimological (Kannel, 1978), clinical (Regan et al, 1977; Ahmed et al, 1975; Rubler, 1975), and pathological (Hamby et al, 1974), studies suggest the existence of a diabetic cardiomyopathy independent of atherosclerosis, coronary artery disease, hypertension, or valvular disease. Diabetic cardiomyopathy implies the existence of a specific diabetic heart disease whose etiology is unknown.

Although the increased mortality rate due to congestive heart failure may be partially due to atherosclerosis it is also due to other factors which are responsible for the development of cardiomyopathy. These include various morphological, metabolic, physiological, and biochemical changes which will be reviewed in the following sections.

Morphology

A variety of morphological changes have been observed in the intramural vessels and ventricular muscle of diabetics. Narrowing or complete obliteration of the lumen of these vessels has been observed in most studies (Blumenthal et al, 1960; Ledet, 1968; Hamby et al, 1974; Regan et al, 1977; Crall and Roberts, 1978), although Ledet (1976) was unable to substantiate severe microangiopathy in the myocardial capillaries of juvenile diabetics.

Associated with these changes are pathological lesions (Giacomelli and Wiener, 1979; Ledet 1976; Rubler et al, 1972; Strobeck et al, 1979), fibrous plaque formation intimal proliferation, calcification (Rubler et al, 1972; Blumenthal et al 1960; Ledet, 1968; Strobeck et al; 1979), and severe hylanization with an increased amount of collagen (Haider et al, 1978; Regan et al, 1977), perivascular tissue, connective tissue, and number of cells in the tunica media (Baandrup et al, 1979; Ledet, 1968). There is a perivascular thickening of the basement membrane, interstitial deposits of glycogen and glycoprotein, (Regan et al, 1974; Regan et al, 1977) and a PAS positive wall seen in many of the diabetics coronary vessels (Haider et al, 1978; Ledet, 1968; 1976; Regan et al 1975; Blumenthal et al, 1960).

Morphological changes in the ventricular muscle include the presence of osmiophillic droplets aligned in rows within the myofibrillar spaces, disrupted sarcomeres, loss of myofilaments, and atrophy of myocytes and amorphous material in the perivascular and interstitial locations (Giacomelli and Wiener, 1979). The number of mitochondria is increased (Regan et al, 1977; Strobeck et al, 1979; Giacomelli and Wiener, 1979), and may be swollen with disrupted cristae and inner mitochondrial membranes (Strobeck et al, 1979; Giacomelli and Wiener, 1979). In addition along with myofibrillar degeneration, an increased deposition of collagen, perivascular tissue and connective tissue is also apparent in the ventricular muscle of diabetics (Ledet, 1968).

Metabolic Changes

Diabetes Mellitus is characterized by the presence of high

circulating glucose levels, and relative insulin deficiency which results in abnormal carbohydrate, protein, and lipid metabolism.

The carbohydrate utilization in hearts of diabetic animals is altered by several mechanisms. The primary dominating effect is reduced glucose transport due to an insulin deficiency (Morgan et al, 1972). The secondary effect concerns changes in phosphorylation kinetics, (eg a seven fold rise in Km), thereby increasing the requirements of intracellular glucose to maintain the same rate of phosphorylation in the diabetic tissue as in the normal tissue (Opie, 1968; Morgan et al, 1961). Changes in the glycolytic pathway in the diabetic state include inhibition of hexokinase (Neely and Morgan, 1981), phosphofructokinase (PFK) due to increased levels of citrate synthetase (Opie et al, 1979; Newsholme and Randle, 1964), and a decrease in pyruvate dehydrogenase activity due to higher tissue levels of aecytl CoA and NADH (Garland and Randle, 1963; Sinclair-Smith, 1979). In addition, increased levels of cardiac glycogen is a common feature of diabetic hearts with any glucose taken up by the cell diverted toward glycogen synthesis (Neely and Morgan, 1981; Shipp and Murthy, 1978; Opie et al, 1979; Penparkgul et al, 1980b). This is a result of the altered activities of the glycogen synthetase (Opie et al, 1979) and phosphorylase system as well as the decreased activity of PFK (Neely and Morgan, 1981). This depression in carbohydrate oxidation could explain the decrease myocardial content of ATP and CP in perfused hearts from diabetic rats (Opie et al, 1979).

I n addition, the rate of glycolysis in the heart is controlled

by the plasma concentration of free fatty acids and ketone bodies as well as cardiac cell lipolysis (Wood et al, 1981). Thus the impaired glycolysis of cardiac muscle in diabetic animals may be the result of increased oxidation of fatty acids and ketone bodies by the myocardium (Randle et al, 1966; Scott, 1975). The concentrations of plasma free fatty acids (FFA) is increased in diabetic animals while tissue levels remain unchanged (Regan et al, 1974). Since the rate and uptake of FFA is proportional to the circulating FFA the uptake of FFA is increased (Opie, 1968), resulting in increased levels of myocardial triglycerides (Scott, 1975; Denton and Randle, 1967; Regan et al 1974; 1975, 1977), cholesterol (Regan et al, 1974; 1975; 1977, Murthy and Shipp, 1977; Evans and Hollenberg, 1964; Haider et al, 1978), and a faster rate of lipolysis (Neely and Morgan, 1981). It has been postulated that the increased activity of the enzyme of triglyceride synthesis may play a role in the accumulation of myocardial triglycerides in diabetes (Murthy and Shipp, 1977). The turnover of tissue triglyceride is also accumulated in hearts of diabetic animals (Evans and Hollenberg, 1964) and increased concentration of acetyl CoA, glyceride, acytylcarnetine (Snoswell and Koundakjian, 1972), cAMP and citrate are found in the diabetic myocardium.

Hearts of normal animals oxidize fatty acids as the major fuel but utilize more carbohydrate immediately following a meal when blood glucose and insulin are elevated. Seventy percent of the ATP is derived from oxidation of fatty acids with essentially all of the remainder derived from glucose oxidation (Neely and Morgan, 1981). In diabetic subjects blood levels of fatty acids and ketone bodies

are high, and these substances account for virtually all of the oxidative fuel of cardiac muscle (Neely and Morgan, 1974), thus virtually all of the ATP is derived from these substances. Therefore, in hearts not working at maximal loads, energy production from oxidation of lipids compensates for reduced glucose use which allows the diabetic heart to maintain normal function. However under maximal stress, an intrinsic defect of the heart to produce ATP seems possible (Sinclair-Smith, 1979). In fact Opie et al, (1979) have demonstrated that promotion of lipid uptake by the heart with regional ischemia tends to exagerate the extent of ischeamic injury .

Abnormalities in ventricular function may also result from imbalances in the rate of synthesis and degradation of specific proteins (Neely and Morgan, 1981). A reduction in the ability of intact hearts from streptozotocin diabetic rats to synthesize protein was found by Pain and Garlick (1974) and Wool et al, (1966) along with an accelerated degradation (Wool et al, 1966). Rannels et al (1970), found that protein synthesis in hearts of diabetic rats proceed at normal rates due to the ability of fatty acids to maintain protein synthesis.

Physiological Changes

Primary physiological abnormalities have been found in clinical and experimental diabetes, both at rest and under various loading conditions.

At rest these abnormalities include a reduced stroke volume (Regan et al, 1977) and increased heart rate (Ahmed et al, 1975)

with no change in cardiac output or coronary flow (Ingebretson et al, 1980). There is an increased conduction time, isovolumic contraction time, atrial diastolic pressure (Ahmed et al, 1975), and endiastolic pressure (Regan et al, 1977), with decreases in the left ventricular ejection fraction (Zoneraich et al, 1977), diastolic compliance (Regan et al, 1978), left ventricular pressure development and rate of rise of ventricular pressure (Ingelbretsen et al, 1980).

In addition, the use of noninvasive measures to measure left ventricular performance in man have revealed several abnormalities in systolic time intervals (STI). These include a shorter left ventricular ejection time (LVET), a prolonged pre ejection period (PEP) and a substantial increase in the ratio of PEP/LVET (Ahmed et al, 1975; Regan et al 1975; Zoneraich et al, 1977). A two hundred percent increase in PEP/LVET is characteristic of the heart in failure by clinical criteria. The forty percent increase found in diabetics is therefore an intermediate between normal and that observed in cardiac decompensation (Ahmed et al, 1975), thus the abnormalities in the left ventricular STI may be considered a preclinical manifestation of cardiac malfunction.

Altered preload and afterload may also contribute if not constitute one of the major determinants of this preclinical abnormality (Regan et al, 1974). During increased preload diabetics develop a significantly higher end diastolic pressure (EDP) than normal controls with similar endiastolic volume increments (Regan et al, 1974). Also apparent with increased preloads are decreases in peak systolic pressure development, cardiac output, (Penpargkul et

al, 1980b; Miller, 1979), stroke work, coronary flow, and peak aortic flow rate (Penpargkul et al, 1980b) with decreases in maximum negative and positive dp/dt (Penpargkul et al, 1980b; Ingebretson et al, 1980). With moderately enhanced afterload the diabetics develop a significantly higher EDP than normal controls but fail to increase EDV as do normal controls (Regan et al, 1974; Regan et al, 1977). Secondary to the abnormal filling of the ventricles due to decreases in EDV are reduced stroke volume with no change in stroke work, and and increased endiastolic filling pressure/volume ratio indicating greater endiastolic wall stiffness (Regan et al, 1974; 1975; 1977). This increased diastolic stiffness in the left ventricular muscle of diabetic dogs supports the greater rise in EDP as compared to controls while the absence of EDV increment can be explained by an altered wall compliance due to accumulation of glycoprotein in the myocardium or impaired ventricular relaxation, (Regan et al, 1974).

Prominent physiological abnormalities observed in the process of relaxation include 1) a delayed onset of relaxation as measured by time to peak isotonic shortening 2) a slowed rate of relaxation characterized by a prolonged time for isometric relaxation and a depressed rate of isotonic and isometric relaxation 3) a delay in reaching peak isometric and isotonic relaxation rates (Fein et al, 1980). Associated with these changes is a depression in the force velocity relation.

Biochemical Alterations

The physiological alterations previously documented most probably result from energy utilizing mechanisms responsible for

contraction and relaxation (Penpargkul et al, 1980b).

A close positive correlation between the Ca^{+2} ATPase activity of actomyosin and the velocity of ventricular muscle contraction has been well established (Barany, 1967). Biochemical evidence indicates that cardiac tissue from diabetic animals show depressed myosin, actomyosin, and myofibril ATPase which may mediate in part the diminished contractility of the diabetic heart (Malhotra et al, 1981; Dillman, 1980; Pang and Weglicki, 1980; Pierce and Dhalla, 1981). In addition Malhotra et al (1981) and Pierce and Dhalla (1981) determined that both basal (Mg^{+2} ATPase) and Ca^{+2}-Mg^{+2} activated ATPase activity are depressed in both male and female diabetic rats, as early as one week after the onset of streptozotocin induced diabetes. Basal activity was lowered from 0.22 to 0.16 umol $P_i/mg^{-1}/5$ min, while Ca^{+2} stimulated ATPase activity was lowered from 0.84 to 0.71 umol $P_i/mg^{-1}/5$ min at 10^{-5} mM Ca^{+2} (Pierce and Dhalla, 1981). Several studies have attempted to determine the causitive relationship between ATPase activity and diabetes in cardiac tissue. Dillman (1980) and Malhotra et al (1981) found a redistribution in the pattern of myosin enzymes V_1, V_2, V_3. The predominating V_1 isoenzyme in control tissue was replaced by the V_3 isoenzyme in diabetic tissue which has a ten fold lower ATPase activity than V_1. The new expression of an isoenzyme of myosin has been suggested to be responsible for altered ATPase activities in various states (Hoh et al, 1977). Pierce and Dhalla (1981), using varying KCl concentrations determined structurally different forms of the protein may be found in diabetic preparations. In addition to these

subtle structural alterations based on ethylene glycol studies they suggest conformational changes at or near the active site of ATPase activity through sulphydryl group modification may inactivate myosin ATPase activity.

Pierce and Dhalla (1981) also indicate that there is no apparent difference in the dependence of myofibrillar ATPase activity on free Ca^{+2} concentration, leading to the possibility that there may be a dependency of the myofibril on free Mg^{+2} concentration.

One of the most prominent physiological alterations is a delay in the rate of relaxation (Fein et al, 1980), leading to the hypothesis that abnormalities exist in the energy utilizing mechanisms responsible for contraction, most probably a disorder of the SR.

Penpargkul et al, (1981) found a reduced Ca^{+2} uptake and a marked depression in SR-Mg^{+2} ATPase and Ca^{+2}-Mg^{+2} ATPase activity in the cardiac SR of streptozotocin induced diabetic rats. The SR functions differed between hearts of male and female rats. The Ca^{+2} uptake was 30% lower in preparations from female rats, while Mg^{+2} and Ca^{+2} ATPase activity was also substantially lower than their male counterparts.

The possibility was considered by Malhotra et al (1981) and Penpargkul et al (1981) that the biochemical alterations were not due to a diabetic effect of streptozotocin on the heart. Studies performed in female rats pretreated with 3-0 methyl glucose a sugar used to prevent the diabetogenic effects of streptozotocin show no depression in Ca^{+2} binding, Ca^{+2} uptake, myofibril ATPase

activity or a delay in the rate of relaxation (Fein et al, 1980; Malhotra et al, 1981; Penpargkul et al 1981). Therefore it has been concluded that streptozotocin injection alone is not sufficient to cause the deliterous effects on the heart, but diabetes must develop for these abnormalities to become manifest.

APPENDIX B

BIOCHEMICAL PROCEDURES AND ASSAY VARIABILITY

A. Isolation of Myofibril Proteins from Cardiac Muscle

Reagents and Chemicals

1. Buffer I: 39 mM Na-Borate; 25 mM KCL; 5 mM EDTA; ph 7.1 (relaxes muscle by removing calcium)
2. Buffer II: 39 mM Na-Borate; 50 mM Tris; ph 7.1 (washes out EDTA)
3. Wash Solution: 50 mM Tris; 10 mM NaN_3 (inhibits mitochondrial ATPase); 100 mM KCL; 0.5% Triton X-100 ph 7.4 (removes SR and sarcolemmal ATPases)
4. Suspension Medium: 150 mM KCL, 50 mM Tris ph 7.4

Procedure

1. Homogenize tissue in 20 volumes of cold Buffer I for 20 seconds at a setting of 7 with a polytron tissue homogenizer (Pt-10
2. Centrifuge the homogenate at 2200 rpm for 12 minutes using a centrifuge
3. Decant supernatant and discard. Resuspend the pellet in 20 volumes of Buffer II, centrifuge atr 2200 rpm for 12 minutes and discard supernatnat
4. Resuspend pellet in 20 volumes of wash solution, centrifuge at 2200 rpm for 12 minutes and discard supernatant
7. repeat step 6
8. Resuspend pellet in 20 volumes suspension medium, centrifuge at 2200 rpm for 12 minutes and discard supernatant
9. resuspend pellet in 7.5 ml suspension medium
10. Take 0.1 ml for protein determination (Lowrey et al. 1951)

B. Protein Determination

Reagents and Chemicals

1. 0.5% Cupric Sulphate ($CuSO_4.5H_2O$)
2. 1.0% Sodium Potassium Tartrate ($NaKC_4H_4O_6$)
3. 5.0% Sodium Carbonate (Na_2CO_3) pH to 12.5 with 10 N NaOH
4. Lowry C Solution: 61.7% $NaCO_3$
   35.7% Deionized Water
   1.3% $CuSO_4$
   1.3% $NaKC_4H_4O_6$
5. Folin Reagent: 1 to 1 (v/v) deionized water
6. 0.3 N KOH
7. Suspension Medium (see section A)

Standard Curve (Protein Stock Solution: 5 mg/ml of bovine serum albumin in distilled water)

Stock (ml)	Buffer (ml)	Concentration (mg/ml)	Final Concentration (ug/ml)
0.0	0.5	0.0	0
0.1	0.4	1.0	6.0
0.2	0.3	2.0	12.0
0.3	0.2	3.0	19.0
0.4	0.1	4.0	25.0
0.5	0.0	5.0	30.0

Procedure

a) Solubilizing Protein

i. Take 0.1 ml of homogenate or standard solution.

ii. Add 0.2 ml of 0.3 N KOH.

iii. Incubate in water bath at 37° for 30 minutes.

b) Reaction mixture

i. Take 0.1 ml (twice) of soluble protein solution from above

ii. Add 5.0 ml of freshly prepared Lowry C Solution to duplicate tubes

iii. Add 0.3 ml of folin reagent of each tube while vortexing. Insure each tube is mixed for the same length of time.

iv. Let reaction mixture stand for at least 45 minutes.

c) Spectrophotometric Analysis

i. Set spectrophotometer wavelength at 750 nm (Pye-Unicam SP8-100)

ii. Use protein blank from the standard curve as the reference

iii. Vortex each sample before the analysis of each sample and record optical density.

Variability of the Protein Determination Procedure

The protein content was determined for repeated samples (n=23) from the myofibril protein of one heart. The coefficient of variation for the Lowry method was 2.98% according to the following formula: $CV = \frac{\text{mean protein content}}{\text{standard deviation}}$

C. Incubation for Myofibril ATPase Activity (Belcastro et al, 1980)

Reagents and Chemicals

1. Reaction Medium (ph 7.0; 30°C)	100 mM KCl
	14 mM Tris
	0.04, 1.0, 10.0 mM $MgCl_2$
2. Calcium EGTA (ph 7.0; 30°C)	1 mM EGTA
	1.2 mM $CaCl_2$
3. 12% Trichloroaecetic Acid (TCA)	
4. 50 mM MgATP	0.1632 g ATP
	0.0508 g $MgCl_2$
	5 ml deionized water
5. Suspension Medium (see myofibril isolation procedure)	

Procedure

A. Reaction Mixture (everything must be kept on ice)

1. Take 0.50 ml of reaction medium into both A (active) and X (non specific activity) tubes.
2. Taking into account the later addition of 0.1 ml ATP solution make each tube up to a total of 1 ml volume with the addition of 0.1 ml of suspension medium.
3. Add 0.05 ml of Ca^{+2} EGTA
4. Add 1.0 ml 12% TCA to every X tube
5. Add 0.25 ml protein (after adjusting to 2 mg/ml) to each tube every 15 seconds.

8. Incubation

1. Vortex each tube and incubate at 30°C for exactly 5 minutes.
2. At 15 second intervals add 0.1 ml of MgATP solution to every tube and allow an incubation time of exactly 5 minutes at 30°C vortex contantly.
3. Add 1 ml TCA to the A tubes at 30 second intervals, and place on ice. At the 15 second mark, remove the X tube and place on ice.
4. Let tubes stand on ice for a minimum of ten minutes.
5. Centrifuge tubes at 2200 rpm for 12 minutes and save supernatant.

Variability of Myofibril ATPase Activity Procedure

The ATPase activity was determined for repeated samples (n=23) from the myofibril protein of one heart. The coefficient of variation for the ATPase method was 1.67% .

D. Phospate Determination

Reagents and Chemicals

1. 10 N Sulpheric Acid: 28 ml H_2SO_4 (98%) made up to 100 ml with deionized water
2. Ammonium Molybdate - Ferrous Sulphate Solution: take 0.5 g ammonium molybdate and compppletely disolve in 5.0 ml 10 N H_2SO_4. Bring up to 30.0 ml with deionized water. Add 2.5 g Ferrous Sulphate and dissolve. Bring up to 50 ml with deionized water.
3. KH_2PO_4 (1mM stock)

Procedure

1. To blank and standard tubes add 2.5 ml 12% TCA
2. To sample tubes (A and X) add 2.0 ml 12% TCA
3. Remove 0.050 ml (50 ul) from standard tubes and replace with 0.050 ml of KH_2PO_4 (phosphate standard)
4. To sample tubes add 0.5 ml supernatant from centifugation
5. Add 2 ml of ammonium molybdate - ferrous sulphate solution to all tubes vortex and let stand for exactly 10 minutes.

Spectrophotometric Analysis

1. Set spectrophotometer to wavelength 800 nm (Pye-Unicam SP8-100)
2. Use deionized water as the reference
3. Record optical density
4. Calculate ATPase activity according to the following equation

$$4 \times .05 \frac{[\text{activated sample OD} - \text{nonspecific activity}]/\text{standard OD}}{\frac{0.5 \text{ mg protein}}{5 \text{ min.}}}$$

E. Calcium Binding to the Myofibril Protein (Solaro and Shiner, 1976)

Reagents and Chemicals

1. Working Solutions:

A) 0.04 mM Mg (pH 7.0)	32.5 mM Na_2ATP
	135.0 mM KCl
	11.3 mM $MgCl_2$
	65.0 mM NaCl
B) 1.0 mM Mg (pH 7.0)	12.3 mM Na_2ATP
	260.0 mM KCl
	16.3 mM $MgCl_2$
	105.0 mM NaCl

2. Calcium Binding Solution 1mCi New England Nuclear
   - 0.415 mM $CaCl_2$
   - 0.500 mM EGTA
   - 5.0 mM Imidazole

3. Calcium-45

Procedure

1. Add 1.0 ml solution A or B to each tube
2. Add 0.99 ml suspension medium to each tube
3. Add 1.0 ml Calcium Binding Solution (5 uM free Ca^{+2})
4. Add 0.01 ml Ca^{45} to each tube. Vortex and preincubate for 3 minutes at 30°C.
5. Add 2.0 ml protein (2 mg/ml) to the A tube. Add 2.0 ml suspension medium to the X tube.
6. Vortex. Incubate for 2 minutes.
7. Stop the reaction by filtering the solution through Millipore HAWP-0.45 micron filters.

8. Add 0.1 ml of filtrate to 5 ml Brays solution and count for 5 minutes.
9. Calculate the Ca^{+2} binding activity with the following equation

$$\text{umol } Ca^{+2}/\text{g myofibril protein} = \frac{\text{1-CPM with protein x 5}}{\text{CPM without protein}} \times .4 \times .02 \div .004 \text{ (protein g)}$$

Variability of Calcium Binding Procedure

Calcium Binding was determined for repeated samples (n=19) from the myofibril protein of one heart. The coefficient of variation for the Calcium Binding method was 8.07%.

APPENDIX C

RAW DATA

Table I: Raw Data for Weekly Body Weights

	Week Number							
Rat Number	1	2	3	4	5	6	7	8
39 (TC)	268	325	337	352	365	379	381	391
64	279	347	346	364	385	406	413	435
49	248	333	340	366	377	383	388	407
66	272	358	373	365	376	392	402	423
67	258	335	326	335	344	356	369	385
44	266	342	359	345	375	400	405	400
42 (SC)	268	348	374	398	415	436	431	475
48	247	327	347	364	381	406	408	421
53	231	312	334	360	377	397	409	440
56	286	370	401	428	448	474	454	483
61	253	351	329	330	356	383	405	425
68	302	378	424	460	482	509	500	567
131 (TD)	234	246	266	272	275	279	258	272
128	228	228	269	275	267	262	233	273
123	235	266	310	328	317	326	297	346
118	236	233	236	211	208	168	184	205
121	236	248	296	308	310	323	298	335
113	251	249	237	302	290	265	261	291
102 (SD)	226	274	294	332	331	321	310	360
127	227	218	253	262	258	239	207	273
108	233	200	252	248	247	220	220	248
105	244	284	310	335	323	306	306	325
137	212	208	270	299	288	269	266	291
111	225	225	271	280	278	257	266	305

Table II: Raw Data for Heart Weight (HW), Body Weight (BW) and HW/BW

Rat Number	Ventricular HW (G)	Body Weight (G)	HW/BW Ratio
39 (TC)	1.025	391	2.62
64	1.229	435	2.83
49	1.146	407	2.82
66	1.229	423	2.90
67	1.056	385	2.74
44	1.086	400	2.72
42 (SC)	1.152	475	2.43
48	1.185	421	2.81
53	1.013	440	2.30
56	1.291	483	2.67
61	1.009	425	2.37
68	1.263	567	2.23
131 (TD)	0.836	272	3.07
128	0.851	273	3.11
123	0.974	346	2.81
118	0.573	205	2.79
121	0.933	335	2.79
113	0.748	291	2.57
102 (SD)	1.026	360	2.85
127	0.746	273	2.73
108	0.775	248	3.13
105	0.977	325	3.00
137	0.872	291	2.99
111	0.815	305	2.67

Table III: Raw Data for Plasma and Urine Glucose Values (mg/dl)

	Plasma Glucose			Urine Glucose	
Rat Number	1 (Wk)	4	8	4	8
42 (SC)	138.5	123.5	124.0	-----	-----
48	125.0	121.0	121.0	-----	61.5
53	136.0	139.0	109.5	-----	200.5
56	120.5	122.5	117.0	-----	-----
61	115.5	134.5	139.0	-----	966.0
68	135.0	131.0	141.5	-----	155.0
92	123.5	116.0	117.0	----	420.0
131 (FD)	648.0	-----	609.0	9009.0	8263.0
128	855.0	735.0	882.0	11392.0	10006.0
123	672.0	672.0	684.0	5376.0	5120.0
118	747.0	984.0	630.0	7255.0	5920.0
121	759.0	537.0	675.0	9229.0	8040.0
113	807.0	786.0	648.0	12190.0	3970.0
102 (SD)	630.0	606.0	723.0	-----	4060.0
127	1101.0	561.0	762.0	-----	7893.0
108	1116.0	717.0	882.0	966.0	8660.0
105	789.0	669.0	780.0	2646.0	5470.0
137	942.0	813.0	570.0	4224.5	9720.0
111	882.0	759.0	831.0	-----	5030.0

Table IV: Raw Data for Myofibril ATPase Activity with Varying Magnesium Concentrations (mM)

	10 uM Calcium			5 mM EGTA		
Rat Number	0.04	1.0	10.0	0.04	1.0	10.0
39 (TC)	0.160	0.150	0.130	0.035	0.033	0.035
64	0.188	0.177	0.167	0.045	0.047	0.032
49	0.163	0.157	0.100	0.037	0.030	0.035
66	0.160	0.167	0.143	0.050	0.048	0.045
67	0.147	0.140	0.105	0.037	0.030	0.022
44	0.135	0.130	0.115	0.050	0.042	0.034
42 (SC)	0.127	0.123	0.102	0.048	0.050	0.050
48	0.130	0.127	0.105	0.045	0.040	0.067
53	0.117	0.112	0.087	0.032	0.035	0.040
56	0.115	0.112	0.100	0.037	0.040	0.065
61	0.112	0.117	0.102	0.023	0.023	0.018
68	0.100	0.100	0.082	0.035	0.027	0.027
131 (TD)	0.062	0.057	0.042	0.018	0.012	0.017
128	0.052	0.050	0.047	0.010	0.010	0.010
123	0.052	0.050	0.042	0.017	0.017	0.015
118	0.053	0.057	0.045	0.018	0.017	0.022
121	0.062	0.061	0.052	0.010	0.010	0.017
113	0.052	0.050	0.045	0.022	0.018	0.022
102 (SD)	0.070	0.067	0.042	0.022	0.022	0.022
127	0.070	0.072	0.055	0.010	0.018	0.018
108	0.067	0.057	0.047	0.025	0.022	0.025
105	0.060	0.057	0.047	0.027	0.025	0.025
137	0.062	0.062	0.048	0.022	0.017	0.013
111	0.053	0.057	0.050	0.027	0.027	0.020

Table V: Raw Data for Calcium Binding (umol Calcium· g-1) Under Varying Magnesium Concentrations (mM).

Rat Number	0.04 mM Mg	1.0 mM Mg
39 (TC)	2.73	----
49	3.14	2.42
66	2.77	2.20
67	3.26	----
42 (SC)	2.56	2.35
53	1.85	2.05
56	----	1.97
61	2.86	2.00
128 (TD)	3.07	2.34
123	3.23	----
118	2.70	3.37
121	2.40	2.82
108 (SD)	2.02	2.49
105	2.57	2.50
137	2.02	2.12

Table VI: Raw Data for the ATPase Activity of the 14 Day Control Group.

Rat Number	1 mM Mg
1	.062
2	.065
3	.060
9	.058
11	.065
12	.073
13	.067
18	.073

Mean = 0.065 $\pm$ .003

Note: The 14 day group has been considered seperate from the TC, SC, TD, and SD groups. Body weight, heart weight, and heart weight to body weight ratios for this group have not been included in the appendix.

APPENDIX D

STATISTICAL PROCEDURES

Table A: Summary of Three Way ANOVA for Myofibril ATPase Activity with Calcium-EGTA

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F ratio
Between Subject	0.11637706		23	
A	0.30257523	0.30257523	1	97.30*
B	0.39949834	0.39949834	1	128.47*
AB	0.39950430	0.39950430	1	128.47*
Subj Within Group	0.62192678	0.31096325	20	
Within Subject	0.74142814		48	
C	0.48639178	0.24319589	2	63.17*
AC	0.12075901	0.60379505	2	1.57
BC	0.44441223	0.22220612	2	5.77*
ABC	0.44512749	0.22256374	2	5.78*
CxSubj Within Gr	0.15400648	0.38501617	40	

A = Control vs Trained

B = Normal vs Diabetic

C = Mg^{+2} Concentrations (0.04, 1.0, 10.0)

Table 8: Summary of Three Way ANOVA for Myofibril ATPase Activity with EGTA

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F ratio
Between Subject	0.10631893		23	
A	0.18046796	0.18046796	1	1.11
B	0.70804693	0.70804693	1	43.63*
AB	0.12798607	0.12798607	1	0.79
Subj Within Group	0.32429695	0.16214847	20	
Within Subject	0.17475486		48	
C	0.36012381	0.18006191	2	0.61
AC	0.15635788	0.78178942	2	2.64
BC	0.43399632	0.21699816	2	0.07
ABC	0.36646053	0.18323027	2	6.19*
CxSubj Within Gr	0.11844635	0.29611576	40	

A = Control vs Trained

B = Normal vs Diabetic

C = Mg^{+2} Concentrations (0.04, 1.0, 10.0)

Table C-A: One Way ANOVA for Training Control With Increasing Magnesium Concentrations

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F ratio	p
Groups	0.37656762	0.00	2	4.39	0.032
Error	0.64311475	0.00	15		

Student-Newman-Keuls post hoc test comparing myofibril ATPase activity at 0.04, 1.0, and 10.0 mM Mg^{+2}

Means	a = 0.159	b = 0.153	c = 0.126
c	3.90*	3.27*	
	(3.675)	(3.015)	
b	0.63		
	(3.015)		

Table C-B: One Way ANOVA for Sedentary Controls With Increasing Magnesium Concentrations

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F ratio	p
Groups	0.16154016	0.00	2	8.21	0.004
Error	0.14761563	0.00	15		

Student-Newman-Keuls post hoc test comparing myofibril ATPase activity at 0.04, 1.0, 10.0 mM Mg^{+2}

Means	a = 0.117	b = 0.116	c = 0.096
c	5.06*	4.86*	
	(3.675)	(3.015)	
b	0.21		
	(3,015)		

Table C-C: One Way ANOVA for Training Diabetic With Increasing Magnesium Concentrations

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F ratio	p
Groups	0.36933110	0.00	2	9.08	0.003
Error	0.30516647	0.00	15		

Student-Newman-Keuls post hoc test comparing myofibril ATPase activity at 0.04, 1.0, and 10.0 mM Mg^{+2}

Means	a = 0.056	b = 0.054	c = 0.045
c	5.61*	4.71*	
	(3.675)	(3.015)	
b	0.91		
	(3.015)		

Table C-D: One Way ANOVA for Sedentary Diabetic With Increasing Magnesium Concentrations

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F ratio	p
Groups	0.86875795	0.00	2	12.72	0.001
Error	0.51216129	0.00	15		

Student-Newman-Keuls post hoc test comparing myofibril ATPase activity at 0.04, 1.0, 10.0 mM Mg^{+2}

Means	a = 0.064	b = 0.062	c = 0.048
c	6.50*	5.80*	
	(3.675)	(3.015)	
b	0.70		
	(3,015)		

Table D-A: Summary of t-tests Comparing Myofibril ATPase Activity Between Trained and Untrained Non Diabetic Animals with Calcium - EGTA

		Control	Trained
	Mean	0.116	0.158
0.04 mM Mg^{+2}	S.E.M.	0.0044	0.007
	DF	6	
	t-value	10.72*	

		Control	Trained
	Mean	0.115	0.153
1.0 mM Mg^{+2}	S.E.M.	0.0038	0.007
	DF	6	
	t-value	7.03*	

		Control	Trained
	Mean	0.096	0.126
10.0 mM Mg^{+2}	S.E.M.	0.0038	0.01
	DF	6	
	t-value	3.52*	

Table D-8: Summary of t-tests Comparing Myofibril ATPase Activity Between Diabetic and Diabetic Trained Animals With Calcium - EGTA

		Control	Trained
0.04 mM Mg^{+2}	Mean	0.064	0.056
	S.E.M.	0.006	0.002
	DF	6	
	t-value	-2.61*	

		Control	Trained
1.0 mM Mg^{+2}	Mean	0.062	0.054
	S.E.M.	0.002	0.004
	DF	6	
	t-value	-2.42*	

		Control	Trained
10.0 mM Mg^{+2}	Mean	0.048	0.045
	S.E.M.	0.002	0.004
	DF	6	
	t-value	-1.52	

Table D-C: Summary of t-tests Comparing Myofibril ATPase Activity Between Control and Diabetic Animals With Calcium - EGTA

		Control	Diabetic
	Mean	0.116	0.064
0.04 mM Mg^{+2}	S.E.M.	0.0044	0.006
	DF	6	
	t-value	26.30*	

		Control	Trained
	Mean	0.115	0.062
1.0 mM Mg^{+2}	S.E.M.	0.0038	0.002
	DF	6	
	t-value	26.06*	

		Control	Trained
	Mean	0.096	0.048
10.0 mM Mg^{+2}	S.E.M.	0.0038	0.002
	DF	6	
	t-value	11.47*	

Table D-D: Summary of t-tests Comparing Myofibril ATPase Activity Between Trained and Untrained Non Diabetic Animals with EGTA

		Control	Trained
0.04 mM Mg^{+2}	Mean	0.035	0.042
	S.E.M.	0.003	0.003
	DF	6	
	t-value	1.28	

		Control	Trained
1.0 mM Mg^{+2}	Mean	0.036	0.037
	S.E.M.	0.004	0.003
	DF	6	
	t-value	0.53	

		Control	Trained
10.0 mM Mg^{+2}	Mean	0.044	0.034
	S.E.M.	0.008	0.0036
	DF	6	
	t-value	-1.62	

Table D-E: Summary of t-tests Comparing Myofibril ATPase Activity Between Control and Diabetic Animals With EGTA

		Control	Diabetic
	Mean	0.035	0.015
0.04 mM Mg^{+2}	S.E.M.	0.003	0.002
	DF	6	
	t-value	2.72*	

		Control	Trained
	Mean	0.036	0.014
1.0 mM Mg^{+2}	S.E.M.	0.004	0.0015
	DF	6	
	t-value	3.356*	

		Control	Trained
	Mean	0.044	0.017
10.0 mM Mg^{+2}	S.E.M.	0.008	0.0018
	DF	6	
	t-value	3.29*	

Table D-F: Summary of t-tests Comparing Myofibril ATPase Activity Between Diabetic Sedentary and Diabetic Trained Animals With EGTA

		Control	Trained
	Mean	0.022	0.015
0.04 mM Mg^{+2}	S.E.M.	0.025	0.002
	DF	6	
	t-value	-3.67*	

		Control	Trained
	Mean	0.021	0.014
1.0 mM Mg^{+2}	S.E.M.	0.0015	0.0015
	DF	6	
	t-value	-11.14*	

		Control	Trained
	Mean	0.020	0.017
10.0 mM Mg^{+2}	S.E.M.	0.002	0.0018
	DF	6	
	t-value	-1.48*	

Table E-A: Summary of t-tests Comparing Heart Weights Between the Groups

	Control	Trained
Mean	1.152	1.128
S.E.M.	0.049	0.036
DF	6	
t-value	0.49	

	Control	Diabetic
Mean	1.152	0.869
S.E.M.	0.049	0.046
DF	6	
t-value	4.90*	

	Diabetic Control	Diabetic Trained
Mean	0.869	0.819
S.E.M.	0.046	0.057
DF	6	
t-value	-0.47	

Table E-8: Summary of t-tests Comparing Body Weights Between the Groups

	Control	Trained
Mean	468.5	406.8
S.E.M.	22.3	7.8
DF	6	
t-value	2.47*	

	Control	Diabetic
Mean	468.5	300.33
S.E.M.	22.3	16.08
DF	6	
t-value	7.82*	

	Diabetic Control	Diabetic Trained
Mean	300.33	287.0
S.E.M.	16.08	20.78
DF	6	
t-value	0.547	

Table E-C: Summary of t-tests Comparing Heart Weight to Body Weight Ratios

	Control	Trained
Mean	2.47	2.77
S.E.M.	0.092	0.040
DF	6	
t-value	3.86*	

	Control	Diabetic
Mean	2.47	2.90
S.E.M.	0.092	0.08
DF	6	
t-value	3.42*	

	Diabetic Control	Diabetic Trained
Mean	2.90	2.85
S.E.M.	0.08	0.082
DF	6	
t-value	0.21	

Table F-A: Three Way ANOVA on Plasma Glucose Levels at 1, 4, and 8, weeks in Diabetic Control and Trained Diabetic Animals.

Source of Variation	Sum of Squares	Mean of Squares	Degrees of Freedom	F ratio	p
Groups	0.13314500	6657.25	2	0.53	0.597
Error	0.18729863	12486.57	15		

Table F-B Summary of t-tests Comparing Diabetic Control and Diabetic Swim Trained Animals

		Control D	Trained D
	Mean	910.0	748.0
1 Week	S.E.M.	76.09	32.0
Plasma Glucose	DF	6	
	t-value	-2.36*	

		Control D	Trained D
	Mean	687.5	742.8
4 Week	S.E.M.	38.50	73.2
Plasma Glucose	DF	6	
	t-value	0.670	

		Control D	Trained D
	Mean	723.3	688.0
8 Week	S.E.M.	66	40.4
Plasma Glucose	DF	6	
	t-value	1.18	

		Control D	Trained D
	Mean	6309.00	6886
8 Week	S.E.M.	83.35	922
Urine Glucose	DF	6	
	t-value	0.071	